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# Report on Five Expert Reviews of the Primedica 2001 Study Report

*(Hormone, Thyroid and Neurohistological Effects of Oral (Drinking Water)Exposure to Ammonium Perchlorate in Pregnant and Lactating Rats and in Fetuses and Nursing Pups Exposed to Ammonium Perchlorate During Gestation or via Maternal Milk, March 2001)*

*Five expert reviews by:*
Victor Denenberg, Ph.D.
Janice Juraska, Ph.D.
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*Prepared for:*
Perchlorate Study Group

May 18, 2001

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## Executive Summary

The Primedica 2001 Study Report (*Hormone, Thyroid and Neurohistological Effects of Oral (Drinking Water) Exposure to Ammonium Perchlorate in Pregnant and Lactating Rats and in Fetuses and Nursing Pups Exposed to Ammonium Perchlorate During Gestation or via Maternal Milk*, dated March, 2001) presents the results of a study to determine whether various doses of perchlorate could cause adverse effects in the offspring of rats exposed prior to cohabitation and during gestation and lactation. The study reported statistically significant changes in some brain morphometry parameters that occurred at low doses of perchlorate. However, these effects did not show a clear dose-response relationship, were not similar in males and females, and were not consistent with the earlier neurodevelopmental study. To aid in the interpretation of this study, the Perchlorate Study Group (PSG) obtained expert review of the neurodevelopmental component of the study. The purpose of this expert review was to obtain advice from recognized experts about the quality and reliability of the experimental methodology and the resulting data for use in determining adverse effects.

We invited five distinguished scientists with expertise in anatomical, endocrinological, and behavioral neurodevelopment to prepare written reviews. Scientists were selected on the basis of their academic and publishing record in topics related to one or more aspects of the work described in the Primedica 2001 Study. The experts are:

- Dr. Victor H. Denenberg (Professor Emeritus, Department of Behavioral Sciences and Psychology, University of Connecticut), a neurodevelopmental psychologist known for his investigations in the field of anatomical and behavioral development of the corpus callosum;
- Dr. Janice M. Juraska (Professor, Division of Neural and Behavioral Biology, University of Illinois), a neurodevelopmental psychologist with expertise in neuroanatomical analyses of brain structures as well as behavioral testing;
- Dr. Pat Levitt (Chair and Professor, Department of Neurobiology, University of Pittsburgh School of Medicine), a neuroanatomist with expertise in developmental neuroanatomy and morphology;
- Dr. Harold L. Schwartz (Clinical Professor of Medicine, University of California at Irvine), a thyroidologist with expertise in developmental hypothyroidism in laboratory animals; and
- Dr. Douglas L. Wahlsten (Professor, Department of Psychology, University of Alberta), an expert in neurodevelopmental behavior and anatomy, design of neurodevelopmental studies, and statistical analyses of such studies.[1]

To help focus their review on factors relevant to use of study results for risk assessment, the experts were provided some background information on the perchlorate project and a charge that included specific questions on the design of the Primedica 2001 Study, the biological significance and plausibility of the results, the relationship of the results to behavioral/ functional effects in adult animals, and generalization of neurodevelopmental data from rats to humans. Each expert worked independently and prepared a separate review. This background document

[1] Dr. Wahlsten submitted his report under his consulting company, MusWare Technology Inc.

and charge is found in Appendix A. The Curricula Vitae for each of the expert reviewers are found in Appendix B. The full reports from each reviewer were submitted to U.S. EPA on May 1, 2001 and are considered to be Appendix C of this report.

The experts raised significant questions and concerns about the sensitivity and specificity of the techniques used to assess neurobehavioral endpoints, the appropriateness of the time periods at which brain tissue was harvested, and other methodological concerns. The following points summarize the responses of the experts in relation to the methodology, the experimental results, and the reliability of extrapolating animal data to humans.

### Adequacy of the Methodology

In general, the experts concluded that the overall experimental design (e.g., treatment of animals, selection of groups) was adequate. However, the experts questioned the reliability and adequacy of the methods used in this study to assess neurodevelopmental endpoints from perchlorate exposure. Specific conclusions regarding the methodology are:

- **No single sectioning plane is optimum for evaluating all brain structures.** For evaluating the corpus callosum and cerebellum, sagittal sections are preferred. For these structures, use of coronal sections may result in inadequate sampling of the structure and introduce between animal variance due to minor differences in the plane of sectioning. However, coronal sections are appropriate for evaluating the cortex and striatum, and horizontal sections are preferred for evaluating the hippocampus.
- **Taking multiple width measures or measuring the area or volume of structures is preferred to the use of single width measures.** Single width measures of brain structures are highly sensitive to precisely where along the brain's anterior-posterior axis the sections were taken but insensitive to changes in cellular parameters (*e.g.*, myelination or cell number).
- **Taking measures from the right and left sides of the corpus callosum could have introduced additional variance.** The corpus callosum is a symmetrical structure; measurements should have been taken at the midline. Since "right" and "left" measurements were reported, these values should be averaged prior to statistical analyses.
- **Pathologists were not blind to the gender and treatment status of all animals.** To eliminate potential bias, pathologists analyzing brain sections should have been blind to the animal's gender and dose level but were only blind to the status of the control and highest dose groups.
- **Post-puberty animals should be evaluated.** At least one post-puberty group should have been evaluated to determine whether changes seen during the neonatal period are temporary or lead to permanent alterations of brain structures. The ages evaluated in this study (postnatal days 10 and 22) are at a time when the neonatal rat brain is undergoing normal neurodevelopmental remodeling.
- **Fixation and sectioning methods introduced between-group variance.** Differences in time between fixation and sectioning for each treatment group and lack of correction for shrinkage in final results added to between-group variance. Asymmetries in coronal

sections are apparent in thumbnail photos of brain sections, particularly for postnatal day 10 pups.

- **Statistical methodologies are inadequate.** The statistical methods used in this study are inadequate to assess the biological significance of potential treatment related effects on any of the morphometric measures.
- **The effects of hypothyroidism on the brain are more complicated than can be detected by measuring the size of brain regions.** Other well-defined endpoints are generally used to evaluate thyroid hormone effects on the brain including target gene mRNA, myelin content, enzyme activity, cellular composition of the corpus callosum, and number of synapses in the hippocampus or cerebellum. For most brain regions evaluated in this study, the literature provides no foundation to suggest that the size of these structures is affected by hypothyroidism.
- **A positive control was not included.** A positive control (*i.e.*, treating a group of subjects with a substance known to induce hypothyroidism) should be included in this study to assess whether reported effects result from hypothyroidism or by some other mode of action.

## Appropriateness of the Results

The experts suggested that no conclusions can be drawn from neurodevelopmental data reported in the Primedica 2001 Study because of the methodological issues discussed above. Because the methods used and ages of pups evaluated in the Primedica 2001 study are non-standard, data from the study cannot be compared with data from control animals in the scientific literature. Specific conclusions are:

- **Hypothyroidism was not induced.** None of the doses of perchlorate appeared to cause hypothyroidism, as suggested by the absence of neurodevelopmental effects reported in the literature to be associated with hypothyroidism. For example, a decrease in the migration of cells from the external granular layer of the cerebellum, a characteristic neurodevelopmental effect of hypothyroidism, was not seen in the cerebellum of postnatal day 10 or postnatal day 22 rats.
- **A clear dose-response relationship is not apparent.** No clear dose-response relationship is apparent for any of the study's neurodevelopmental endpoints. For example, there is a lack of consistency between doses that produce the most significant effects on thyroid parameters and doses that produce the most significant changes in linear dimensions of brain structures.
- **No relationship has been established between changes in brain morphometry and behavioral/functional effects.** Studies of brain morphometry in animals and humans have not established a direct cause-and-effect relationship between changes in brain structure dimensions and behavioral/functional effects in adults.

### Significance of Findings in Rats to Expected Outcomes in Humans

Overall, the peer reviewers conclude that effects observed in rats in appropriately conducted experiments might be observed in humans. Specific conclusions are:

- **Rats are an appropriate model for human neurodevelopment.** If properly measured effects are seen on neurodevelopmental endpoints in rats, then effects might be expected in humans.
- **Species differences can affect extrapolation to humans.** Confounding factors, including species differences in the phases and rate of brain growth and differences in maternal care, could affect extrapolation of results to humans.

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## Introduction

The Primedica 2001 Study Report (*Hormone, Thyroid and Neurohistological Effects of Oral (Drinking Water) Exposure to Ammonium Perchlorate in Pregnant and Lactating Rats and in Fetuses and Nursing Pups Exposed to Ammonium Perchlorate During Gestation or via Maternal Milk*, dated March, 2001) was submitted for review by five experts in the field of neurodevelopment.

The goal of the Primedica Study was to evaluate the effects of perchlorate on the thyroid in dams and fetuses at multiple time points in order to generate data for a pharmacokinetic model and to establish a NOAEL for thyroid effects in neonatal pups. In addition, another goal was to repeat aspects of the Argus 1998 neurodevelopmental study to evaluate possible adverse effects of perchlorate in dams on brain development in pups. Thyroid histopathology and thyroid hormone levels (TSH, T3 and T4) were measured in dams on gestation day 21 and postnatal days (PND) 10 and 22, and in pups on the same days plus PND 5. Morphometric measurements of certain brain regions were made in pups on PND 10 and 22. In general, increasing doses of perchlorate increased the serum levels of thyroid stimulating hormone (TSH) and decreased levels of the thyroid hormones $T_4$ and $T_3$ in dams and pups. Thyroid hyperplasia was observed in dams at higher doses; no effects on thyroid histopathology were observed in pups. Some effects on brain morphometry in pups were also reported.

The Primedica Study Report concluded that linear measures of a variety of forebrain structures, taken in the coronal plane, demonstrate statistically significant changes in size at various exposure levels. Gender analysis revealed a trend towards increases in males and decreases in females. While this analysis revealed statistically significant changes at some levels of exposure, no clear dose-response effect with perchlorate exposure was seen, raising questions about the biological significance of the morphometry findings. Therefore, the PSG assembled a group of experts to review the report, focusing on data regarding neurodevelopmental endpoints. In the following sections, the experts are introduced, their reviews are summarized, and each expert's review is presented in its entirety.

### Conduct of the Expert Review

In order to provide additional insight into how these results could be most appropriately interpreted and used, the Perchlorate Study Group (PSG), assembled a group of experts to review the Primedica 2001 Study report. The experts were asked to focus their review only on the neurodevelopmental component of the study and the aspects of the thyroid effects as they pertain to neurodevelopment. The experts were not asked to provide a complete review of the entire study, nor were they necessarily informed of the multiple goals considered when designing the study.

The experts were provided with a background document describing the history of the perchlorate project and a charge to reviewers that listed several specific questions for the reviewers to consider. The review materials provided to the reviewers included the draft final report of the Primedica 2001 study (minus the individual animal tables), thumbnail photographs of the brain sections from the PND 10 and 22 pups, plots of the dose-response curves for the morphometric measurements, key portions of the Argus 1998 neurodevelopmental study, and a summary of the Bekkadahl 2000 motor activity study. The background document, charge to reviewers, and complete list of review materials is presented in Appendix A. The reviewers were asked to spend approximately 20 hours reviewing the documents, assessing the methodology and outcomes of the Primedica 2001 Study, and preparing a written report. Given the limited time for their review, experts addressed what they considered to be the most important aspects of the Primedica 2001 Study.

Individually, each scientist was selected because he or she has expertise in one or more scientific specialties relevant to this experiment, and publishes extensively in these areas. As a group, these scientists have expertise in all the neurodevelopmental endpoints addressed in the Primedica 2001 Study. The scientists were asked to confine their reviews to those aspects of the report that pertain to their own expertise; Curricula Vita are provided in Appendix B. The expert reviewers were:

- Dr. Victor H. Denenberg is a neurodevelopmental psychologist known for his investigations in the field of anatomical and behavioral development of the corpus callosum; his review focused primarily upon measures of the corpus callosum.
- Dr. Janice M. Juraska is a neurodevelopmental psychologist with expertise in neuroanatomical analyses of brain structures as well as behavioral testing.
- Dr. Pat Levitt is a neuroanatomist whose research encompasses the fields of developmental neuroanatomy and morphology.
- Dr. Harold L. Schwartz is a thyroidologist with expertise in developmental hypothyroidism in laboratory animals.
- Dr. Douglas L. Wahlsten is an expert in neurodevelopmental behavior and anatomy, the design of neurodevelopmental studies, and statistical analyses of such studies.

The reviewers' overall conclusions are summarized below. Subsequent sections compile and summarize the reviewers' responses to the specific questions presented to them in the guidelines for review. The complete final review report from each reviewer was submitted to U.S. EPA on May 1, 2001, and these final reviews are considered to be Appendix C of this report.

### Overall Conclusions Regarding the Primedica 2001 Study

The overall goal of the Primedica 2001 Study was to determine if perchlorate, at the administered doses, causes adverse effects in the offspring of rats exposed prior to cohabitation and during gestation and lactation. As the report relates to neurodevelopmental endpoints, each expert offered overall conclusions regarding the experimental methodology, the resulting data, and the extrapolation of the data from animals to humans. All five reviewers concluded that issues related to the design of the neurodevelopmental component of the study prevented drawing any conclusions about the effects of perchlorate on neurodevelopment.

> *There is insufficient sampling of the CC* [corpus callosum] *to draw any meaningful conclusions about the effects upon the offspring of exposing pregnant rats to perchlorate in the drinking water.* (Denenberg, p. 4). ... *it is not possible to make any meaningful interpretation of the empirical findings* (Denenberg, p. 2).

> *While these comparisons revealed statistically significant changes at some levels of exposure, there is no clear dose-response effect with perchlorate exposure, making it very difficult to determine whether the data are biologically significant. The statistical tests used may not be adequate to resolve whether the relationships are biologically significant, and it is strongly recommended that a statistician whose expertise extends to such biological systems is consulted* (Levitt, p. 2). *Overall, the morphometric data do reflect consistent effects of perchlorate on the structural development of select structures in the forebrain...* but *...it is more likely that the induced morphometric changes by perchlorate are due either to normal variation in the measure used in the Primedica study, or to complex effects on molecular characteristics that were not measured in this study* (Levitt, p. 3).

> *Morphometry based on thickness measures in coronal sections is acceptable for judging thickness of the cerebral cortex, but not for evaluating changes in the size of the corpus callosum, cerebellum, or hippocampus. By far the weakest part of the study was the statistical analysis of the data...Lacking such an analysis, I cannot form an opinion on the central issue of whether perchlorate significantly altered brain morphology or what is the minimal effective dose.* (Wahlsten, p. 17).

> *Given what appear to be major weaknesses in the neurohistological data, including lack of dose response, sex dependent differences and lack of reproducibility, it is not possible to conclude there is any effect of perchlorate at these doses on brain development.* (Schwartz, p. 7).

> *It is clear from Primedica 2001 that the effects of perchlorate do not dramatically alter development. However, the results of Primedica 2001 are inconclusive with regard to more subtle effects that are needed for the calculation of a Reference Dose of perchlorate* (Juraska, p. 1).

## Reviewer Responses to Specific Questions

The charge to reviewers included a list of questions to help focus the experts' reviews. The following sections summarize the reviewers' responses to the questions in five general areas: study design, morphometric methods, experimental results, relationship of results to behavioral effects, extrapolation from rats to humans. As stated earlier, since each scientist has expertise in a slightly different specialty of neurodevelopment, not all scientists answered each question.

## 1. Comments Regarding Study Design

The reviewers were asked to comment on various aspects of the experimental design. Most reviewers provided positive comments about the general study design (*e.g.*, breeding of females, administration of doses, selection of pups for harvesting tissue). However, all of the reviewers indicate serious concerns about the methods used to assess neurodevelopmental endpoints. Major areas of concern included that even the highest dose of perchlorate was insufficient to produce hypothyroidism, the method used to fix brain tissue, the lack of blinding regarding treatment groups during histological analyses, the method of evaluating morphometric data, and the age of offspring when tissues were harvested. Specific comments about the experimental design are summarized below.

### 1.1 General Experimental Design

Several reviewers comment that the general design of the experiment, with regard to establishing treatment groups and handling animals, is appropriate.

> *The descriptions of the protocols followed in breeding females, selecting which females to use, selecting pups, etc., all appear quite sound. The Ns of 13-16 per group is certainly within acceptable limits .... The use of one male and one female per litter is excellent* (Denenberg, p. 11).

> *The animal husbandry and methods of administering the substances were conventional and appeared to be adequate*" (Wahlsten, p. 1). *In my opinion, the design and execution of the Primedica study up to the point where brain histology was done were adequate and in some ways superior, especially with regard to the long term administration of the perchlorate* (Wahlsten, p. 16).

### 1.2 Perchlorate and Hypothyroidism

Dr. Schwartz, the only thyroidologist among the reviewers, comments that the doses of perchlorate did not cause hypothyroidism in the pregnant rats or their offspring.

> *Were these animals, in fact, hypothyroid? I believe the answer is clearly, NO. It is well known that hypothyroidism leads to a reduction in the growth rate of both dams, fetuses and pups, measured as change in body weight .... There was no difference in these parameters among any of the treatment groups at any time point. Added evidence against hypothyroidism in the dams is the lack of an effect on food intake. Hypothyroid adult rats generally reduce their food intake 20% or more .... Further, litter size is consistently reduced by as much as 50% in hypothyroid dams .... It was also noted in the earlier study (Argus) that, at the doses used, behavioral development was unaffected. As far back as the studies of Eayrs and colleagues...it was noted that in hypothyroid pups there was*

*retardation of learning and of development of behaviors such as the righting reflex and the startle response* (Schwartz, p. 4-5).

*In my laboratory and in the literature generally... the concentration of potassium perchlorate used to induce hypothyroidism in laboratory animals is 1-2% (10 to 20mg/ml) in the drinking water. In a rat weighing 250gm drinking about 30ml per day this is a dose of 1.2 to 2.4gm/kg/day as much as 80-fold the maximum 30 mg/kg/day dose used in the current study. It is not clear on what basis the doses of perchlorate used in this study were chosen* (Schwartz, p. 5).

**<u>1.3 Fixation and Embedding of Tissue</u>**

Three experts comment on the methods used for fixation of brain tissue.[2] One reviewer comments that the method was adequate, another inadequate, and the third states that because the method used causes shrinkage, correction for shrinkage of brain tissues measures should be done.

Dr. Levitt comments that fixation method was adequate:

> *The methods for tissue processing were well-controlled and resulted in the maximal homology of brain section quality.... Immersion fixation used here does not provide the optimal cellular preservation, but introduced minimum variation in fixation quality* (Levitt, p.8).

Conversely, Dr. Juraska comments that the fixation method was inadequate:

> *There are technical inadequacies to the present study...*" that include "*...fixation of the brains. The groups were not sectioned at the same time .... This could affect the size of various components of the brains .... Time between fixation and sectioning must be held constant between the groups* (Juraska, p. 1).

Finally, Dr. Wahlsten comments that the fixation method is adequate, he states that paraffin embedding can cause shrinkage of the tissue that should be adjusted for in final reported values.

> *Brain tissue ... was fixed by immersion in 10% neutral buffered formalin, a method that is inferior to intracardiac perfusion but is nevertheless adequate and is much quicker .... Paraffin embedding ... usually shrinks the tissue by a very large amount, reducing linear measures by almost 40% of their true values in some tissue .... This ... is not a major cause for concern, provided the investigators take care to determine the degree of shrinkage and then correct their measures to restore them to valid figures. In the Primedica study, however, thickness measures were not corrected for shrinkage .... Consequently,*

[2] Brains were fixed by immersion in 10% neutral buffered formalin. PND 10 and 22 brains were then shipped to Consultants in Veterinary Pathology (CVP) and Experimental Pathology Laboratories, Inc. (EPL), respectively, where the brains were sectioned and embedded in paraffin. Groups I and V were sectioned initially, and then the blocks from the other groups were sectioned in a stepwise fashion. CVP performed the morphometric measurements on both the PND 10 and PND 22 slides (Primedica, 2001).

*comparisons with measures in the published literature may not be valid* (Wahlsten, p. 3).

**1.4 Blinding**

The Primedica 2001 Study reports that morphometric measurements were performed blind (*i.e.*, the pathologist did not know the sex or treatment group of the animal) for slides from Group I (control) and Group V (30 mg/kg-d dose) offspring only. Measurements in the remaining treatment groups were not evaluated blind (CVP, 2001b, p. 4). Both Drs. Juraska and Wahlsten comment that lack of blinding is unacceptable:

> *This is not acceptable. The bias of the experimenter, often unintentional, cannot be allowed* (Juraska, p. 1).

> *The person making morphometric measures of brain tissue should always be blind with respect to treatment, sex and even age of the animals being assessed. It is not clear that an acceptable level of blind assessment was achieved in the Primedica study .... This is a very peculiar procedure and is clearly inadequate* (Wahlsten, p. 2).

**1.5 Handling of Pups**

Dr. Levitt comments that the handling of pups[3] could have affected morphometric parameters, with possibly different effects in males and females:

> *Postnatal handling of pups and lactating dams adds a confound that could affect brain development and maternal care .... Limited handling (as little as 15 minutes of separation of pups from dam during the first postnatal week of life) is sufficient to alter gene expression ... and response to stress. Maternal care (licking) is altered significantly by the handling as well .... A new study should eliminate handling effects by using parallel litters that are not assessed behaviorally or morphologically, but used instead to monitor weight*" (Levitt, p. 5). *It is possible that the handling ... had different effects on males and females .... There is evidence that such early postnatal handling can alter stress hormone responsiveness in females, but not males* (Levitt, p. 12).

## 2. Comments on Appropriateness of Morphometric Methods

The reviewers were asked to comment on the morphometric methods used[4] to evaluate adverse effects on brain development offspring.

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[3] Pup body weights were measured daily (Primedica, 2001).

[4] Twenty-one to 25 morphometric measurements were taken from each brain. Two measurements (anterior to posterior length of the cerebrum and anterior to posterior length of the cerebellum) were taken from the intact brains prior to trimming and processing. The other measurements were taken from coronal sections, embedded in paraffin.

**2.1 Appropriateness of Using Single Linear Measurements of Brain Tissue**

Most reviewers suggested that taking multiple width measures or measuring the area or volume of brain structures is preferred to the use of single width measures. Single width measures of brain structures are highly sensitive to precisely where along the brain's anterior-posterior axis the sections were taken but insensitive to changes in cellular parameters (*e.g.*, myelination or cell number)

> *No one uses a single width score as a meaningful measure* (Denenberg, p. 3). If coronal sections are used, ... *it is necessary to obtain multiple coronal slices along the anterior-posterior axis to ensure adequate sampling throughout the CC* (Denenberg, p. 11).
>
> *The area of the corpus callosum should be taken, not thickness. Area is more likely to be proportional to the cellular measures that are ultimately of interest - axon number and myelination. A structure might not vary in thickness but can still vary in area.* (Juraska, p. 2).
>
> *... this reviewer recommends against using linear measures to look for crude changes in the size of specific structures as a sensitive measure for documenting effects of thyroid hormones or TSH. Instead, the study design should have included sufficient power to allow for other quantitative methods of cell-based assays* (Levitt, p. 7; emphasis in original).
>
> *... thickness measures are sensitive to functionally irrelevant and minor changes in the shape of a structure as well as more important alterations in its overall size .... Measures of area of a structure in a slice are superior, and volume compiled from successive slices is even better for some brain regions* (Wahlsten, p. 3). *The* [cross sectional area of a sagittal section of the corpus callosum] *is expected to be directly proportional to the number of axons connecting the cerebral hemispheres and their degree of myelination .... Thickness, on the other hand, is strongly dependent on precisely where along the anterior-posterior axis the coronal section is made* (Wahlsten, p. 4).

Dr. Wahlsten also outlines several other problems with measuring the thickness of other brain structures in coronal sections. Regarding the external granular layer (EGL) of the cerebellum, for example:

> *For the cerebellum, thickness of one coronal section is prone to error because of the angle of the slice and inherent variation in the EGL itself. A sagittal section, on the other hand, lends itself readily to a measure of cross-sectional area of the entire cerebellum and also allows useful measures of the thickness of different zones of the cerebellar cortex...Thickness of the rat cerebral cortex, on the other*

For most measures, measurements were taken on the right and left sides of the midline of a coronal section, and right and left measurements are presented separately in the text of the report (Primedica, 2001).

*hand, is generally an acceptable index, provided thickness is measured at several locations throughout the cortex ...* (Wahlsten, p. 4).

**2.2 Choice of Sectioning Plane**

The reviewers indicated that no single sectioning plane is optimum for evaluating all brain structures. For evaluating the corpus callosum and cerebellum, sagittal sections are preferred. For these structures, use of coronal sections may result in inadequate sampling of the structure and introduce between animal variance due to minor differences in the plane of sectioning. However, coronal sections are preferred for evaluating the cortex and striatum, and horizontal sections are appropriate for evaluating the hippocampus.

> *Several methods have been proposed for obtaining CC* [corpus callosum] *measurements. All these procedures start with a sagittal section of the callosum...* (Denenberg, p. 3).
>
> *A mid-sagittal measurement would also be preferable to the coronal section* (Juraska, p. 2).
>
> *This* [use of coronal sections] *is adequate for analysis of cortex and striatum. This plane of section, however, introduces serious problems in matching anatomical level for analysis of corpus callosum and hippocampus .... The former is best evaluated in the sagittal plane, the latter in the horizontal plane .... Cerebellum is best analyzed in the sagittal plane* (Levitt, p. 9).
>
> *Sagittal sections are preferable when conducting morphometric studies of corpus callosum... Dorsal hippocampus is best studied with the aid of coronal sections, whereas ventral hippocampus requires the use of horizontal sections to visualize the sizes of CA1, CA3 and anatomical layers...* (Wahlsten, p. 7-8).

**2.3 Issues Regarding the Bilateral Measurements of Brain Tissues**

Drs. Denenberg and Juraska comment that the appropriate approach for measuring the corpus callosum is to take measurements at the midline.

> *There is no 'right' or 'left' corpus callosum .... The proper measurement of the CC is at the midline of the coronal section, using a minimum of 10 sections to obtain a series of measures* (Denenberg, p. 3; emphasis in original).
>
> *... the tightly packed axons and glia seen at the midline become diluted with neurons and processes oriented in all directions once the pathway enters the brain. Hence, a midline measurement of the structure is essential* (Juraska, p. 2).

Given that separate right and left measures were collected for the corpus callosum in this study, Drs. Denenberg and Wahlsten suggest that the right and left measures should be averaged:

*Even though the measurements are flawed in this study, it might be possible to obtain some information, since the measures obtained would be expected to be proportional to the correct data* (Denenberg, p. 4).

*Measuring both halves of a bilaterally symmetrical structure is an acceptable practice, but these measures should be averaged for analysis, not analysed separately .... Considering measures separately on the left and right inflates the error variance and makes the ANOVA less sensitive to genuine treatment effects, if they are present* (Wahlsten, p. 4).

Both Drs. Levitt and Wahlsten note asymmetries in coronal sections, apparent in the thumbnail photos. The reviewers suggest that asymmetries could cause measurements on the right and left side of the midline to be different without any biological basis:

*The use of the coronal* [sic] *plane only is likely to introduce inconsistencies due to section asymmetry and the inherent difficulties in matching rostro-caudal levels across brains between treatment groups. Side asymmetries...* [are] *the most likely reason for the inexplicable side effects of some linear measure* (Levitt, p. 5-6).

*... it was obvious from the thumbnail sketches of coronal sections that in many brains, especially in the DL10 rats* [day of lactation 10], *the section was not cut perpendicularly to the midline of the brain .... in many instances, especially in the DL10 brains, the actual anterior-posterior levels of ostensibly the same sections were quite different... and this would have given rise to substantial differences between animals within a group in thickness of the corpus callosum, for example* (Wahlsten, p. 5-6).

## 2.4 Issues Regarding Shrinkage of Brain Tissue and Other Factors Introduced during Processing

Several of the reviewers are concerned about variability introduced by differential shrinkage of brain tissues during fixation and embedding.

*Paraffin embedding ... usually shrinks the tissue by a very large amount, reducing linear measures by almost 40% of their true values in some tissue .... Thickness measures were not corrected for shrinkage of the tissue during histological processing, and no indication of the degree of shrinkage was provided* (Wahlsten, p. 3).

The reviewers also suggest that operator error, asymmetric sectioning, and group differences between time of sectioning all contribute to observed variance, but that variance appears to be within acceptable limits.

*All morphometric analyses have inherent sources of variation, but the variation in the means in the report of linear measures are within expectation for experimental error .... Based on the appearance of the sections in the thumbnails provided, it appears that the interbrain variation in shrinkage and sectioning quality is low, thus reflecting very well-controlled processing methods. There is variability across measures (±4-20%), and this is likely due to differential tissue shrinkage, operator error in making morphometric linear measurements across different brain samples, and inherent difficulties in matching anatomical location because of asymmetric sectioning in the coronal plane. The variability is generally ≤10%, which is routine for the experimental design used here* (Levitt, p. 7-8).

*The groups were not sectioned at the same time .... This could affect the size of various components of the brains .... Time between fixation and sectioning must be held constant between the groups* (Juraska, p. 1).

**2.5 Limitations in Evaluating Rat Pups at PND 10 and 22**

The reviewers were asked if the methods used and the time periods selected to assess the developing brain of treated offspring were appropriate for to answer the questions raised during the design of this experiment The reviewers agree that a limitation of this study is that a group of offspring from at least one post-puberty age is needed to assess permanent effects to offspring of perchlorate treatment.

*Measures at two time points (Days 10 and 22) is not sufficient to determine a developmental path .... It will be necessary to obtain brain measures at least at one post-puberty age to get an estimate of a relatively stable adult brain* (Denenberg, p. 12).

*... the appropriate end point for morphological analysis was not included in the study (sexually mature rats), which is necessary to eliminate the possibility that the measured changes are simply due to a modest developmental delay* (Levitt, p. 3).

*The DL22 rats examined in this study had not yet been weaned and were far from being mature. Consequently, it is possible that treatment effects of perchlorate altered the rate of development but had no influence on the eventual, mature size of brain structures* (Wahlsten, p. 2).

Several reviewers provide examples of structures that are undergoing significant changes at the ages examined in this study, suggesting that taking measures at PND 10 and 22 might result in considerable variability in results and a lack of understanding of perchlorate effects on some stages of neurodevelopment.

In he corpus callosum, "*... myelination is not complete in the rat until at least 40 days of age.* (Denenberg, p. 12).

*... rapid changes in brains at DL10 and DL22 make the results sensitive to changes in rate of growth as opposed to later asymptotic levels. Myelination of the CC in particular is rudimentary at DL10 and far from complete at DL22, although major anatomical changes brought about by elimination of axons are past* (Wahlsten, p. 8).

*... we have found that axons continue to withdraw from the splenium of the corpus callosum after day 25 and myelination is just starting at day 25 .... Neuron death (apoptosis) continues in the female visual cortex to at least day 25 ... and hormonal manipulations after day 20 change the number of neurons in the adult visual cortex... There is also dendritic growth and regression between days 30 and 60 in the visual cortex ... and days 43 to adulthood in the somatosensory cortex .... Likewise, synapses increase 1.5 fold between days 21 and 41 in the dentate gyrus of the hippocampus ...* (Juraska, p. 2-3).

## 2.6 Comparison With Values in the Literature

The reviewers were asked to comment on how the linear measurements of brain structures taken from the control animals in this study compare with the same structures in literature.
Most of the reviewers wrote that they are not aware of any studies that present data similar to those measured in the Primedica Study, either because the methods used in the Primedica study are unconventional or because other studies have not examined rats at these ages.

*Even when the CC values are corrected, I am not aware of any studies measuring CC width at or around 10 and 22 days. I don't know about the other brain measures at these ages* (Denenberg, p. 11).

*Given the idiosyncratic methods employed in this study and the specific ages examined, comparisons with the published literature are difficult. The rat brain is changing rapidly during the DL10 to DL22 period, and it would be important to compare brains no more than one or two days different in age .... I was surprised to find no references to any published literature on the rat brain in the entire report* (Wahlsten, p. 7-8). *There are some published data on thickness of the CC at midline ... but I am not aware of studies in which measures were taken bilaterally at the same distance from midline .... Ventral hippocampus requires the use of horizontal sections to visualize the sizes of CA1, CA3, and anatomical layers. Probably the best morphometric data in the literature involve horizontal sections.... Direct comparisons of thicknesses in terms of microns (μm) is rendered difficult by the use of paraffin embedding that causes substantial tissue shrinkage ...* (Wahlsten, p. 7).

*Linear measures provided are comparable to values in the literature (Bayer and Altman, 1991 for comparative sectioned material at P10 rat)* (Levitt, p. 9).

## 3. Comments Regarding the Experimental Results

The reviewers comment on the experimental results reported in the Primedica 2001 Study. They were asked if these results could be relied upon to determine adverse effects of perchlorate exposure and whether the scientific literature supports these results.

### 3.1 Hypothyroidism and Neonatal Brain Tissues

***Literature Support for a Relationship Between Hypothyroidism and Effects on Brain Structures***

The reviewers were asked if the results in the Primedica 2001 Study are consistent with effects observed in the literature. The reviewers state that they are unaware of any studies of hypothyroid animals that report findings similar to the Primedica 2001 study. In addition, reviewers note that brain changes associated with hypothyroidism reported in the literature are not seen in this study.

> *Literature searches failed to identify studies in which states of hypothyroidism were induced during development and measurements were performed on the same forebrain regions showing changes in the Primedica 2001 study* (Levitt, p. 17)

> *Hypothyroidism results in developmental delays, reduction in size of cerebellum due to altered cell proliferation and cell death and reduced myelination (see Koibuchi and Chin, 2000 for review). None of these changes are consistently seen in the Primedica study* (Levitt, p. 17).

> *I know of no data in the literature going back 50 years related to the actions of these hormones in brain that describes a difference in the biology of the thyroid hormones between the sexes or of differences in response from one side of the brain to the other* (Schwartz, p. 6). *... there is no foundation in the literature suggesting that the size of most of these areas is affected by hypothyroidism. Further, none of the well-defined endpoints of thyroid hormone affect in brain, target gene mRNAs, myelin content, enzyme activities, etc that have been so well described over a fifty year period was included as control* (Schwartz, p. 7).

The reviewers gave several examples of brain changes that would have been expected as a result of hypothyroidism:

> *One of the most well described effects of hypothyroidism in the newborn pup is the delayed development of the cerebellum. In the euthyroid rat pup, the external granular layer disappears by PND24. In hypothyroid pups the width of the external granular layer is greater than in euthyroid pups... Yet no difference in the cerebellar external granular layer was seen in this study* (Schwartz, p. 6).

> *It is possible that a toxin might substantially delay myelination or the migration of granule cells from the external granular layer (EGL) of the cerebellum .... EGL thickness should be measured at an age that is midway between the age of its peak size and the first age when it disappears altogether. Rats at DL22 would likely be near the age when the EGL normally disappears and therefore would be able to reveal only major, grossly abnormal retardation* (Wahlsten, p. 2).

Dr. Wahlsten writes that the methodology used in the current study would not have been able to detect changes in brain structures that have been reported in hypothyroid rats. He provides several examples of changes in brain structures associated with hypothyroidism that were not seen in this study.

> *Postnatal deficiency of thyroid hormone ... is known to increase the size of the cerebellum and the number and complexity of its folia, fissures, and sulci in the rat brain .... These phenomena are readily perceived in a sagittal section through the middle of the cerebellum but are almost impossible to measure in a coronal section ...* (Wahlsten, p. 4-5).

> *The number of myelinated axons is substantially reduced in the anterior commissure and corpus callosum of hypothyroid rats .... A reduction of militated axons will ... reduce the cross-sectional area of the corpus callosum ... whereas large changes in the numbers of unmyelinated axons may have little impact on corpus callosum area .... Recent evidence indicates that thyroid hormones are involved in the maturation of oligodendroglia, cells that are responsible for the formation of myelin in the central nervous system ...* (Wahlsten, p. 5).

***Consistency Between Effects on the Thyroid and Changes in Brain Structures***

The reviewers were asked if the changes observed in linear dimensions of brain tissue are consistent with the thyroid endocrine animal literature. The reviewers noted a lack of consistency between the doses associated with the most significant effects on thyroid hormone levels and doses associated with the most significant changes in brain structures. They also noted that even though blood thyroid hormone levels were altered, the study results do not provide evidence that a clear hypothyroid state was induced.

> *The most effective doses of perchlorate that induce thyroid hormone deficiencies overlap with, but are not identical to those concentrations that resulted in reproducible changes in 'brain structure. The failure of the highest dose of perchlorate to induce structural changes, in the context of reproducible alterations in TH* [thyroid hormone] *levels, indicates that if the changes in brain structures are biologically relevant, there must be other interacting elements driving the changes* (Levitt, p.18).

> *Clearly, these doses were sufficient to affect hormonal synthesis, although at best, the changes in plasma hormone levels observed were modest ...* " (Schwartz, p. 4),

then elaborates, "*The data ... suggest that the intake of perchlorate was not sufficient to induce a hypothyroid state even at the 30mg dose. Unfortunately, no accepted tests of thyroid state were done to test this issue. Thus, it is concluded that any changes observed in the brains of these animals are likely not the result of reduced tissue T3 concentrations. It would have been of value to have measured the tissue T3 concentrations* (Schwartz, p. 7).

## 3.2 Consistency of Perchlorate Effects on Brain Development

The reviewers were asked if the data from the Primedica (2001) study and the Argus (1998) neurobehavioral study demonstrate a consistent pattern of adverse effects on neurodevelopment and behaviour of pre and postnatally exposure offspring. The majority of the reviewers did not respond to this question. However, Dr. Schwartz observed that the conclusions of this study are inconsistent with those from the previous Argus (1998) study:

> *The reduction in brain weight in the perchlorate groups seen in the earlier study was not duplicated in the current round at any dose. Again, the increase in size at PND12 in the cerebellum earlier seen at 3mg was not seen at any dose in this study. In the earlier study, an increase in the size of the corpus callosum was seen at 10 mg but not at the 3 mg dose at PND12* (Schwartz, p. 6).

## 3.3 Biological Significance of Changes in Linear Dimensions

The reviewers were asked to comment on whether the statistically significant changes in linear dimensions observed in this study should be considered biologically significant, and, if not, what degree and/or direction of change (*i.e.*, 10% increase, 20% decrease, etc.) would be considered biologically significant. While Dr. Denenberg suggested that it would be prudent to assume that any significant change was biologically significant, in general the reviewers concluded that a variety of methodological issues preclude using the data to draw any conclusions regarding whether the findings are biologically significant.

> *... it is not possible to make any meaningful interpretation of the empirical findings* (Denenberg, p. 2) *With respect to neuromorphology, the corpus callosum data are largely useless except to suggest that this fiber bundle needs to be examined carefully .... Are these biologically relevant? First, the experiment and analyses must be designed and conducted correctly. Then I think one has to assume relevance for any change in a brain region that is known to play a key role in behavior* (Denenberg, p. 10).

> *It is clear from Primedica 2001 that the effects of perchlorate do not dramatically alter development. However, the results of Primedica 2001 are inconclusive with regard to more subtle effects that are needed for the calculation of a Reference Dose of perchlorate* (Juraska, p. 1).

> *While these comparisons revealed statistically significant changes at some levels of exposure, there is no clear dose-response effect with perchlorate exposure,*

> *making it very difficult to determine whether the data are biologically significant .... The statistical tests used may not be adequate to resolve whether the relationships are biologically significant ...* (Levitt, p. 2). *Overall, the morphometric data do reflect consistent effects of perchlorate on the structural development of select structures in the forebrain*", but "*It is more likely that the induced morphometric changes by perchlorate are due either to normal variation in the measure used in the Primedica study, or to complex effects on molecular characteristics that were not measured in this study* (Levitt, p. 3).

> *Given what appear to be major weaknesses in the neurohistological data, including lack of dose response, sex dependent differences and lack of reproducibility, it is not possible to conclude there is any effect of perchlorate at these doses on brain development* (Schwartz, p. 7).

> *Lacking such an analysis* [sufficient statistical analyses], *I cannot form an opinion on the central issue of whether perchlorate significantly altered brain morphology or what may be the minimal effective dose* (Wahlsten, p. 16).

Most of the reviewers comment that determining the biological significance of any change in the linear dimensions of a brain structure is difficult, even when statistically significant, and that determining the biological significance of outcomes of the current study is particularly difficult due to problems with the morphometric methods and statistical analyses. For example, Dr. Juraska commented that even in well-conducted studies, size measurements can be inconclusive and are difficult to interpret as an indicator of biologically significant effects:

> *The ultimate question is ... if structure is altered. Size is being used as a substitute for cellular and biochemical measures .... Size measurements can be inconclusive because more than one cellular effect is occurring. For example in the corpus callosum, the number of axons may be increased (due to disruptions in axon withdrawal) while myelination may be decreased. Thus there might be no effect on size of the structure even though callosal function may be compromised. Such complicated effects make interpretations of size measurements difficult during development when several different cellular events are occurring asynchronously* (Juraska, p. 2).

> *Well-respected clinical studies do not use linear measures to discern biologically significant alterations in brain development... a major deficiency in the Primedica study is the fact that there is not a linear relationship between change in the size of brain structures and the qualitative or quantitative extent of functional deficits* (Levitt, p. 13). *One can hypothesize about the extent of size change that would be needed to correlate with serious brain dysfunction ... volume and areal changes in brain structures in the 10-20% range, which are statistically significant, also can be biologically significant. As one example, the brain weight of children with autism, at autopsy, is approximately 10% larger.... Cerebellar size is increased to a similar extent .... In another example, the*

*increase in ventricular size in schizophrenia is 8-12%, with a similar decrease in hippocampal volume ...* (Levitt, p. 13).

Dr. Schwartz commented that biological significance cannot be inferred due to a lack of dose-response:

> *A lack of dose-response is evident throughout these studies both for any given brain segment and among brain segments. Although, generally plasma hormones were lowest in the 30mg dose group, most often this group showed no treatment effects. Nor is there consistency of the effects among doses from one study to another or, in the current study, from one brain segment to another*" (Schwartz, p. 6).

Dr. Wahlsten provides substantial comments about the statistical analyses, stating that the methods used were inadequate to establish a treatment effect:

> *This piecemeal approach does not allow a proper statistical evaluation of sex or age differences in treatment effects* (Wahlsten, p. 1). *The analysis of data in this report was not adequate to allow me to evaluate the consistency of perchlorate effects on any measure. A fair number of significant deviations from the zero-dose control were detected, particularly for male rats, so it would be imprudent to dismiss the report as failing to demonstrate any effect on brain morphometry. Examining the means ... suggests to me that perchlorate effects on morphometric measures were not large, with the possible exception of cortex and maybe hippocampus of DL22 males* (Wahlsten, p. 10). *The report cites numerous P values but gives no indicators of effect size* (Wahlsten, p. 12). *How great a change in thickness should be required to support a claim of harm or benefit to the organism? This is a matter that should be agreed upon in advance by the sponsors and consumers of the research, and it should play the crucial role in deciding the sample size in order to confer sufficient power on the test* (Wahlsten, p. 11).

### 3.4 Gender Differences

The reviewers were asked if the developmental literature provide support for the apparent differences between males and females in the direction of size changes reported in the Primedica 2001 Study. Several reviewers suggest that the mechanism of action of perchlorate may differ between the sexes, and that sex hormones may be involved. However, other reviewers comment that these apparent sex differences may be the result of methodological problems with how the data were collected or with inadequacies in the statistical analyses.

> *The most obvious answer is that the sex hormones are involved and that perchlorate is having different effects upon a testosterone background than upon an estrogen background. With respect to the corpus callosum, there are interesting sex differences in the distribution of myelinated and unmyelinated fibers .... An EM study will be necessary in a future study* (Denenberg, p. 12-13).

> *There is no known explanation* [for the differences in males vs. females]. *There is however, a possible explanation that would require direct testing .... It is possible that the differences in males and females measured here is a consequence of differential gene regulation in males compared to females, perhaps due to an additional regulatory element that has great activity in developing female brain. Literature searches did not identify studies examining effects of TH on genes also mediated by estrogen* (Levitt, p.12).

> *Numerous studies in rats have found a sex difference in overall CC size and a variety of steroid hormone effects on the CC* (Wahlsten, p. 15).

> *Since these data are highly suspect, I do not think much can be said about consistency until the study is re-done (and re-done correctly)* (Denenberg, p. 10).

> *... a piecemeal approach to data analysis whereby separate tests of effects are done for males and females is not an adequate basis for judging a sex difference in response. ...I see no clear evidence that there was a decrease in thickness measures with increasing dose for females .... Lacking compelling statistical evidence, I can see no point in delving deeply into the physiology of the sexes* (Wahlsten, p. 10).

**3.5 Shape of Dose Response Curve**

The reviewers were asked if the literature supports larger effects at intermediate doses compared to higher doses of a hypothyroid inducing agent. In general, the reviewers agreed that a U-shaped dose-response curve is possible in biological systems. The reviewers commented, however, that the range of doses in this study was probably not sufficiently wide for a U-shaped dose-response to be seen, and that improved statistical analyses should be conducted to test whether a U-shaped dose response exists. As stated earlier, Dr. Schwartz believes that no dose of perchlorate caused hypothyroidism in dams or offspring.

> *Most biological functions will be U-shaped (or inverted-U shaped) over a wide enough range of an independent variable .... However, it is not certain that a wide enough range of perchlorate doses has been built into the study to speak confidently about U-shaped functions* (Denenberg, p. 12).

> *Statistical analysis should be done on the original data in order to determine whether this pattern is stochastic or a real biological phenomenon* (Levitt, p. 10), but ... *intermediate doses of a particular agent may produce a more substantial biological response than low or high doses* (Levitt, p. 11).

> *An intermediate dose could certainly have the maximum effect when there is some kind of biphasic dose-response relationship on certain measures, but it could also be spurious. The proper test of a U-shaped dose-response curve is the quadratic term in a regression equation, assessing whether addition of an* $X^2$ *term*

*significantly improves the fit of the equation to the data. No such test was reported* (Wahlsten, p. 10).

## 4. Comments Regarding the Relationship with Behavioral/Functional Effects

The reviewers were asked if alterations in brain structures during development or once offspring are mature can be associated with abnormal behavioral/functional effects. Experts were also asked if appropriate behavioral testing was conducted for structures suspected of being adversely effected by a hypothyroid inducing agent. The reviewers' comments in these areas are summarized below.

### 4.1 Relationship Between Changes in Morphometric Measures and Behavioural Effects

The reviewers were asked if the statistically significant changes reported in the Primedica 2001 Study regarding the linear dimensions of offspring brain regions are likely to be associated with behavioural/functional effects in adult animals. The reviewers agree that existing data do not support a cause and effect relationship between changes in morphometric measures of brain structures and behavioral/ functional effects, in either rats or humans.

Dr. Denenberg comments that the behaviors usually studied in the rat will not likely be reflected in changes in morphometry of the corpus callosum: *For the corpus callosum, even when the proper measurements are made, it is most unlikely that the behaviors usually studied in the rat will be affected by callosal morphology .... Balogh et al. failed to find any relationship between the behavioral measures and callosal status* (Denenberg, p. 13).

*... It is important to emphasize that while there may be patterns to what was assessed here, these measures* *<u>are not amenable</u>* *to direct cause-and-effect evaluation with the neurobehavioral study* (Levitt, p. 10; emphasis in original). *... no studies with which this reviewer is familiar have determined a well-defined correlation between the extent of changes in the linear dimensions of the size of certain brain structures and functional effects in adult animals. It is possible, but it is more likely that an underlying cellular change would be responsible for any behavioral deficits .... Changes in linear measures are caused by alterations in cell structure or organization ... any attempt to define a relationship between putative behavioral changes and structure would need to include the assessment of the specific cell populations and circuits responsible for mediating a particular function* (Levitt, p. 18-19).

*In the literature of human neuropsychology, many claims have been staked on a relation between brain size and intelligence or IQ, but the data point to a very small correlation with almost no explanatory impact ...* (Wahlsten, p. 14). Regarding the corpus callosum, *... it has been very difficult to demonstrate any reliable behavioral correlate of modest changes in CC cross-sectional area ... Humans lacking a CC from the beginning of cortex formation tend to be a bit* *clumsy and are on average about 10 points below average on IQ tests, but they almost always suffer other neurological challenges...and there is no indication that reduced cognitive functioning arises specifically from the lack of a CC .... My conclusion ... is that one would not expect any major change in behavior to arise from a modest change in the thickness of the CC in one or two coronal sections. I would not recommend dismissal of a thickness change as irrelevant, however. It is possible that a change in thickness might serve as an indicator of a more functionally important change elsewhere in the nervous system* (Wahlsten, p. 14-15).

**4.2 Appropriateness of Behavioral Endpoints**

The reviewers were asked if behavioural testing of offspring cited in Argus (1998) and Bekkedal (2001; Appendix C) were appropriate for the effects of hypothyroidism on the developing brain. Dr. Levitt comments that he believes that the appropriate behavioral endpoints were evaluated (Levitt, p. 19). Dr. Denenberg reviewed the earlier Argus 1998 study and concluded that the range of behavioral endpoints tested was narrow:

*The only behavioral data are from the Argus 1998 study. No effects were found for passive learning, an 'M-test' for water maze learning, or acoustic startle. Greater motor activity was obtained in an open-field type of test.*[5] *However, this effect apparently has not been replicated in the Primedica 2001 Study, though additional statistical analyses are yet to be done* (Denenberg, p. 10). *With the exception of the 'M-maze,' the other tasks are familiar ones in a behavioral laboratory. However... the sampling of possible behaviors represented by these tests is very narrow. For example, no measures were obtained of spatial learning ... non-spatial associative learning (e.g., discrimination learning), fear-based active learning (one-way or two-way shuttlebox), Pavlovian learning, novelty/exploratory behavior, or spatial and non-spatial working memory, to name a few important behavioral processes* (Denenberg, p. 10).

## 5. Comments Regarding Significance of Findings in Rats to Expected Outcomes in Humans

The reviewers were asked if developmental effects observed in the offspring of maternally treated rats would be expected to be observed in humans; in other words, would they consider the rat to be an appropriate model to predict human effects. Most of the reviewers comment that if effects are seen in a properly conducted study in rats, then effects might be suspected in humans.

[5] The experts were not given statistical reanalyses of these data. When appropriate statistical reanalyses were conducted on these data, statistical significance was not evident for any treatment group (Goodman, 1999).

> *If the corpus callosum, when properly measured, yields significant effects for the perchlorate treatment, this is likely to be found in humans as well* (Denenberg, p. 14).

> *While there are many well-conserved events of brain development that are regulated by the same genes and proteins across species, the effects of perturbing the milieu in which the brain develops may or may not result in the same changes across species. It is possible that the brain effects of perchlorate in rats could occur in humans as well. It also is possible that it may not have any effect on brain size …* (Levitt, p. 19).

> *If researchers find that a compound has consistent effects across a wide range of mammalian species, then I think we can be confident that results will generalize to humans, but I would not care to generalize on the basis of data from any one species. If toxic effects are observed in rats, this would certainly provide grounds for worrying about possible harm to humans* (Wahlsten, p. 15).

However, Drs. Levitt and Wahlsten also suggested some confounding factors that might affect extrapolation between species.

> *A … confound in using the rat as a model of human neurodevelopment are the inherent differences in maternal care that are known to affect brain development … and the possible differences in perchlorate metabolism that may exist between rodents and humans* (Levitt, p. 20).

> *Species differences in development may be important when we want to match phases of brain growth …. Humans require many years to progress from weaning to sexual maturity, whereas rats can breed only a few weeks after weaning. The DL10 rats in the Primedica study had just begun to benefit from myelination of the corpus callosum, a stage that occurs prior to birth in humans. The DL22 rats were only a few days from weaning, something that commonly occurs one year after birth in humans but can be delayed for several years for social reasons* (Wahlsten, p. 16).

## References

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# Appendix A: Background Document and Charge to Reviewers

# Review of Brain Morphometry Results From a Perchlorate Toxicity Study (Primedica 2001)

**March 5, 2001**

**Sponsored by the Perchlorate Study Group**

**Toxicology Excellence for Risk Assessment**
**1757 Chase Avenue**
**Cincinnati, OH 45223**

## Introduction

Thank you for assisting in this interesting and important process. We have asked several experts in the fields of neurodevelopment and developmental thyroid endocrinology to prepare an expert review of the study entitled *Hormone, Thyroid and Neurohistological Effects of Oral (Drinking Water) Exposure to Ammonium Perchlorate in Pregnant and Lactating Rats and in Fetuses and Nursing Pups Exposed to Ammonium Perchlorate During Gestation or via Maternal Milk* (Primedica, 2001). In this study, perchlorate, a known anti-thyroid agent, was given to female rats prior to and during gestation and lactation in order to assess the effects of perchlorate on rat fetuses and pups. Thyroid hormone status and thyroid pathology was evaluated in the dams and pups. In addition, the effects of perchlorate on brain histopathology and morphometry was evaluated in pups on postnatal days (PND) 1
0 and 22. Although no effects were observed on brain histopathology at any dose level, some statistically significant differences in the linear dimensions of certain brain regions were observed in some groups of treated pups compared to controls at both time points. We are seeking your guidance on interpreting the significance of these results and on whether conclusions about the effects of perchlorate exposure on rat brain development can reliably be reached from these results. This study and your review will be submitted to the U.S. EPA for consideration in their risk assessment of perchlorate.

The U.S. EPA is in the process of developing regulations in the form of a drinking water standard for perchlorate to protect public health. The Primedica (2001) study was conducted to provide information to support this process and your review will contribute to an understanding of how this study should be used in the risk assessment. Although further research will undoubtedly be done on perchlorate, the urgency of the public health concern requires the U.S. EPA to move forward expeditiously with its review and regulatory process. That process will probably be concluded before any additional research is completed. We ask that you keep this context in mind during your review, and that you comment specifically on what can be concluded from the existing studies and data.

In the next section, we provide you with some background information on perchlorate, the U.S. EPA risk assessment methods and on other health effects studies that have been conducted in order to provide some context for your review. In the final section, we provide guidelines for reviewers, asking a series of specific questions to help frame your review. Please review this background information and guidelines thoroughly before proceeding with your review as it puts your review in context of the larger project.

## Background Information

### What is Perchlorate?

Perchlorate is an ion, $ClO_4^-$, that has been used both industrially and medically. Large-scale production of perchlorate-containing chemicals began in the U.S. in the mid-1950s. About 92% of perchlorate manufactured is used as a solid rocket propellant, 7% is used in explosives, and about 1% is used in other products such as fireworks and airbag inflators. Perchlorate was used from the 1950s to mid 1960s to treat Graves's disease, an autoimmune disease that results in hypothyroidism. However, this medical use declined after a limited number of patients developed severe hematological side effects after being treated with very large doses (>1200 mg/kg-day) of perchlorate. Currently, perchlorate is used in conjunction with the heart drug amiodorone to prevent iodine toxicity.

Perchlorate was first detected in a groundwater system in the United States in 1985 and then again in 1992. In 1997, an improved analytical method reduced the detection limit from 100 ppb to 4 ppb in water, and subsequently, perchlorate was detected in groundwater supplies in many states. The sources of the groundwater contamination are its production and use as a rocket fuel, as well as naturally occurring perchlorate in some nitrate fertilizers. Because of the potential for exposure to large numbers of people through their drinking water, the EPA initiated assessment and regulatory activities aimed at developing an appropriate drinking water standard for perchlorate adequate to protect public health.

Preliminary assessments in 1992 and 1995 found that the toxicological database in existence at the time was inadequate to properly evaluate possible public health risks. Although human case reports and short-term animal studies in the literature at that time suggested that perchlorate had effects on thyroid iodine uptake, circulating thyroid hormones, and thyroid pathology, there was very limited information on possible effects on other organs or on effects at low doses. To provide the minimum data needed to conduct a risk assessment, a cooperative effort was initiated by the U.S.EPA, the Department of Defense, and the Perchlorate Study Group, a coalition of 9 private companies that make or used perchlorate, to design and fund needed toxicology studies. By the end of 1998, the following studies had been completed:

- A study of 90-day oral administration to rats found effects on thyroid hormones and mild thyroid histopathology, but no other effects.
- A Segment II (standard teratology protocol; gross structural and visceral exam of fetuses 1 day before delivery) developmental study in rabbits found thyroid effects, but no developmental effects.
- A 2-generation reproductive study found effects in the thyroid, but no effect on reproduction.
- A neurodevelopmental study in rats found effects on circulating thyroid hormones and on thyroid histology. Also observed were changes in motor activity at PND 14 that were not statistically significant, but showed a trend, and statistically significant changes in brain morphometry in male pups at PND 12. The latter measurement was observed in a non-random sampling of culled pups and was not part of the original design, but these potential effects raised concerns and were the basis for the rationale for further brain developmental study. This study is discussed in more detail below.
- Genotoxicity studies found no evidence of direct effects on DNA.
- Immunotoxicity studies found no clear evidence of effects but design flaws led to repeated studies.

In December 1998, U.S. EPA released a document that reviewed these studies as well as the historical literature on perchlorate effects and presented a preliminary risk assessment (U.S.EPA, 1998[6]). A peer review panel reviewed the EPA document in February 1999 and made recommendations for additional studies[7]. Among the studies recommended is the Primedica 2001 study which you are being asked to review. U.S. EPA is currently considering this new data as it finalizes its risk assessment document.

## What are U.S. EPA Risk Assessment Methods?

Central to the EPA approach to non-cancer risk assessment is the development of a Reference Dose (RfD) for perchlorate. The RfD is rooted in the assumption that a threshold exists for systemic effects, so a range of exposures from zero to some finite value can be tolerated by the organism with essentially no chance of expression of the toxic effect. A reference dose is an estimate of a dose to a sensitive human population that is likely to be without adverse effects during a lifetime of exposure. Since such a dose level is never known with certainty; the EPA uses a standardized approach to evaluation of the data that is available to derive the RfD. Specifically, the approach involves a review and integration of all relevant information and the identification of a No-Observed-Adverse-Effect Level (NOAEL) or a Lowest Observed Adverse Effect Level (LOAEL) from existing studies.

It was expected that the studies of perchlorate toxicity outlined above could be used in the process of defining a NOAEL or a LOAEL for a critical effect of perchlorate exposure. A NOAEL is defined as the *highest* exposure level at which there is no statistically or biologically significant increase in the frequency or severity of adverse effects between the exposed population and its appropriate control. A LOAEL is defined as the *lowest* exposure level at which there are statistically or biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control group. The U.S. EPA defines a "critical effect" as the first adverse effect, or its known precursor, that occurs to the most sensitive species as the dose rate of an agent increases. For the purposes of identifying critical effects to be applied in establishing RfDs, the U.S. EPA defines an "adverse effect" as a biochemical change, functional impairment, or pathologic lesion that affects the performance of the whole organism, or reduces an organism's ability to respond to an additional environmental challenge.

The RfD is derived by dividing the appropriate NOAEL by an uncertainty factor (UF). Generally, the UF consists of multiples of 10, each factor representing a specific area of uncertainty inherent in the available data. For example, a factor of 10 may be introduced to account for the possible differences in responsiveness between humans and animals in prolonged

[6] U.S. EPA 1998. Perchlorate Environmental Contamination: Toxicological Review and Risk Characterization Based on Emerging Information. NCEA-1-0503, Office of Research and Development. U.S. Environmental Protection Agency, Washington D.C. December 31, External Review Draft

[7] U.S. EPA 1999. Perchlorate Peer Review Workshop Report. EPA Contract Number 68-W98-085, Office of Solid Waste. U.S. Environmental Protection Agency, Washington D.C. May 31.

exposure studies. A second factor may be used to account for variation in susceptibility among individuals in the human population. The uncertainty factors may be modified from the default value of 10 based on the data relevant to the specific area of uncertainty.

The determination of the appropriate NOAEL and critical effect are made after an integrative review of all relevant toxicological studies. The determination evaluates the consistency of the effects documented in toxicity studies with anything that is known about the mechanism of action of the chemical in question. The analysis considers the consistency of the effect across species and across sexes, if such information exists. It also considers the consistency across different doses in a single or in multiple studies. Consistency of effects across studies and with the mechanism of action lend more support to the choice of the critical effect and study to use as the basis for the risk assessment.

**How Have EPA Risk Assessment Methods Been Applied to Perchlorate Data?**

Imperative in developing a risk assessment is determining what effects should be considered "adverse." The series of studies of perchlorate that were completed in 1998 found effects on thyroid hormones at doses as low as 0.01 mg/kg/day in the 90 day drinking water study, and thyroid gland hypertrophy at 0.1 mg/kg/day. Effects on the thyroid were found consistently across several of the studies and were consistent with the known mechanism of action of perchlorate as an inhibitor of iodine uptake into the thyroid, with subsequent affects on thyroid histology (e.g., hypertrophy and hyperplasia) and on circulating thyroid hormones and TSH. A significant point of discussion at the peer review of EPA's 1998 risk assessment was the determination of what effect on thyroid function would be considered an adverse effect to the organism, in light of the known capacity of the thyroid to adapt to changing conditions. In their report, the peer review panel concluded that the normal function of the thyroid is to maintain thyroid hormone levels and the normal thyroid can respond to environmental conditions, such as iodine deficiency, with substantial physical changes that work to maintain homeostasis. These changes alone do not constitute an adverse effect. The peer review panel stated that hypertrophy and/or hormone changes are part of the normal functioning of the thyroid. They recommended that hyperplasia should be considered an adverse effect and used as a basis for RfD derivation. Thus, the peer review panel provided fairly clear guidelines as to what effect in the thyroid should be considered adverse.

Primarily as a result of this direction, and the fact that hyperplasia in the thyroid occurred only at higher doses of about 3-10 mg/kg/day, the possible developmental effects on the brain have assumed great importance in the evaluation of perchlorate risk. The known effect of thyroid hormones and TSH on brain development, and the data from the Argus (1998) study, discussed in the next section, raised concerns about a potential secondary effect of perchlorate exposure on brain development. Because of the importance of this effect for identifying a potentially "most-sensitive population", and a "critical effect", it was imperative to determine whether these effects occur reproducibly, whether they are biologically significant, and if so, at what doses they occur. Because the initial neurobehavioral developmental study (Argus, 1998) was not adequate to answer these questions, based on the judgement of the peer review panel, the Primedica (2001) study, which is the subject of this review, and a repeat of the motor activity tests (Bekkedal, 2000) were performed. For historical perspective, the Argus (1998) study is described in the following section.

## What Do the Health Effects Studies Show?

Neurobehavioral developmental study (Argus, 1998; see Attachment B)

*Design.* In 1997, a panel of perchlorate scientists was convened to decide on the study designs required to provide necessary data for risk assessment. Based on the recognized effects of thyroid hormones on brain development, learning, and behavior, including the effect of T4 deficit on IQ and learning ability in later life, the review group determined that neurobehavioral endpoints were the most important endpoints. The study was designed primarily to evaluate a battery of behavioral outcomes, and relatively little thyroid assessment was planned. (The thyroid was being amply evaluated in the other concurrent studies, listed previously, and designed at the same time.) The emphasis was placed on functional studies, and neuropathology was only done in relatively small groups of animals (n=6) at 2 time points (postnatal day [PND] 12 and PND 82-85). The behavioral tests were done according to a standard EPA design. One modification was to start dosing at gestation day 0, rather than the typical dosing on GD6 in the rat, in order to allow time for hypothyroidism to develop.

*Results and Conclusions.* There were changes in the thyroid gland and in thyroid hormone levels in the limited evaluation of these endpoints that were made (Attachments B-2 and B-3). The behavioral tests showed no effects on passive avoidance, watermaze test, or auditory startle response (Attachment B-7). However the motor activity test found statistically significant increased activity in males at PND14 in 3/18 time blocks, but the total activity was not significantly different (see Attachment B-8). There were no statistically significant effects on total motor activity in females or at other time points in males. The EPA considered the motor activity data in male pups at PND14 to be possibly biologically significant because of the magnitude of the change although the measurement was highly variable and not statistically significant. As a result of this concern a better motor activity test was designed and carried out in 2000 (Attachment C).

Standard neurohistology found no abnormalities in control or high dose animals at PND 12 (Attachment B-5) or PND 82-85 (Attachment B-6). The morphometric measurements that were initially made only in the control and high dose groups found increase in size of the corpus callosum that was statistically significant in females and was increased but not statistically significant in males (Attachment B-6, Tables 1 and 2). There were also some statistically significant changes of unknown biological significance at PND82-85 (no change in corpus callosum; Attachment B-6, Tables 1 and 2). Based on these suggestions of an effect, EPA requested brain morphometry on the next lower dose group (3 mg/kg/day), for PND12 pups only.

The results shown in Attachment B-4 showed that the effect on the corpus callosum was not evident at the 3 mg/kg/day dose group, but several other effects were present at the lower dose but not at the higher dose. The EPA concluded that these results could be indicative of an adverse effect on brain development.

*Comments from 1999 Peer Review Panel.* The 1999 peer review panel recommended that neurological endpoints that are expected to be affected by thyroid hormones, such as corpus callosum and other myelinated structures, should be examined to assess an agent that primarily affects the thyroid gland. It was noted that the caudal portion of the corpus callosum is more likely affected, and the section taken in the Argus (1998) study did not adequately evaluate the corpus callosum. It was pointed out that the behavioral measures used are not well characterized in terms of thyroid hormone effects, except motor activity, and that motor activity is a valuable endpoint to examine more closely. Other endpoints mentioned by the peer reviewers included measurement of specific mRNA's known to be affected by thyroid hormones, measurement of apoptosis in cerebellar granular layer cells, and detailed EM study of corpus callosum cell numbers and volumes. The peer review group also recommended that dosing should begin at least 2 weeks before mating to allow hypothyroidism time to develop.

<u>The Primedica 2001 study (see Attachment A)</u>
*Design.* The design of the Primedica (2001) study was a collaborative effort of U.S. EPA, U.S. DOD, with some assistance from National Institute of Environmental Health Sciences. The designers of the study were cognizant of the results of the earlier study and the comments from the peer review committee. The study design was somewhat constrained by competing uses of the data. The thyroid hormone data was needed to support development of a physiologically based pharmacokinetic model for pregnant and lactating rats and the overall design reflected this requirement, in part. An additional constraint was the need to have the design follow a standardized protocol as closely as possible so that it could be done by a contract laboratory. For this reason, many of the measurements that had been suggested by the peer-reviewers were considered to be too experimental and not sufficiently standardized. The final experimental protocol used a standard EPA protocol, with some additional examination of the corpus callosum. The study was designed to ensure consistent and well-documented brain sectioning. The details of the design can be found in the draft final report (Attachment A-1) and the neuropathology report (Attachment A-2, A-3).

*Results.* This section presents a summary of the thyroid effects observed in the study in both dams and fetuses/pups in order to present background information for your review of the brain morphometry effects. The details of the brain morphometry results can be found in the draft final report (Attachment A-1) and the neuropathology reports (Attachments A-2 and A-3). No effects were observed on maternal clinical signs, food and water consumption, body weight, or any litter or natural delivery observations at any time point. In dams, relative thyroid weight was significantly increased in the high dose group at all time points. Incidence of thyroid hypertrophy was increased only in the high dose group at all time points. Incidence of thyroid hyperplasia in dams was not increased in any dose group on gestation day (GD) 21. Incidence of

thyroid hyperplasia in dams was increased in the high dose group on PND 10 and in both the 1 and 30 mg/kg-day dose groups on PND 22. In dams on GD 21, TSH and T4 were significantly increased in all dose groups and T3 was significantly increased only in the high dose group. In dams on PND 10 and 22, TSH was significantly increased in all dose groups, T4 was increased only in the high dose group, and T3 was not increased in any dose group.
In male pups, thyroid weight was significantly increased in the high dose group on PND 5, in all dose groups on PND 10, and at doses ≥ 1.0 mg/kg-day on PND 22. In female pups, thyroid weight was not increased on PND 5, were increased only in the high dose on PND 10, and were increased at doses ≥ 1.0 mg/kg-day on PND 22. Thyroids from GD 21 fetuses were not weighed. No increased incidence of thyroid hypertrophy or hyperplasia was observed at any time point in any dose group in either male or female pups. In general, thyroid hormones were statistically significantly increased in at least one dose group at each time point (except T4 on PND 10 and T3 on PND 5); however no consistent patterns were observed. TSH was increased at doses at doses ≥ 1.0 mg/kg-day on GD 21, PND 5 and PND 22 female pups; in the high dose group only on PND 10; and in all dose groups in PND 22 male pups. T4 was significantly increased in the high dose group on GD 21 and in all dose groups on PND 5 and PND 22 male pups. T3 was significantly increased in all dose groups on GD 21, at doses ≥ 1.0 mg/kg-day on PND 10 and PND 22 male pups, and in the high dose group only in PND 22 female pups.

<u>The Motor Activity Study (Bekkedal, 2000; see Attachment C).</u>
The motor activity study (Bekkedal, 2000) was designed specifically to provide an improved study of the effects of exposure to perchlorate during gestation on development of motor activity in rats. This study was designed to repeat and extend the findings on motor activity in the Argus, 1998 study. This study has been completed, but its public release has not been approved by U.S.DOD at this time. . The study PI has informed us that repeated measures ANOVA found no difference in activity between the treatment groups. However, the analysis of this data by the U.S. EPA has not been completed and it is possible that other analyses may come to different conclusions.

**<u>Guidelines for Reviewers</u>**

Your review should focus on the brain morphometry results of the Primedica 2001 study, considering other aspects of the study as they affect the interpretation of the brain morphometry. The Argus (1998) study is also included for your review, but we are not asking for a detailed review of the methodology and results in the Argus (1998) study. Please use it to the extent that you find it useful to address the specific questions below. Also, please note that the two studies were conducted by the same laboratory, which underwent a name change between 1998 and 2001. The U.S. EPA risk assessors are currently completing a revised risk assessment report of perchlorate, which will undergo a peer review in the summer of 2001. The Primedica (2001) has been submitted to U.S. EPA, and your comments will also be submitted U.S. EPA to facilitate their understanding of the results of this study. Therefore, as you provide your comments, please keep in mind the overriding questions that are important to risk assessors:

Do the statistically significant changes observed in the Primedica (2001) study, when considered in light of the results of the Argus (1998) neurobehavioral developmental study, represent a consistent effect of perchlorate exposure on behavioral or structural development of the brain?

If these studies do demonstrate a consistent effect, is the effect biologically relevant?

If the observed effects are biologically relevant, can they be considered adverse, as defined in the discussion on page 3 of this cover paper?

If the effect on brain development or behavior is considered adverse, what dose produces the adverse effect in the test species (rat)?

If the effects are considered to be adverse, are they relevant to humans?

To the extent possible, please orient your comments to address these issues using the existing studies.

We would like the review to be conducted consistent with the manner in which you would typically conduct a peer review of any scientific study. We are interested in knowing whether the research and results presented in this study constitute a reliable indication of adverse effects due to perchlorate exposure. If you feel some data are reliable and some are not, please make that distinction. In reviewing the study, please point out any errors or information that you identify as omitted, misleading, or unclear. Keep in mind that key statements of fact, unless well known, should be supported by literature references and/or experimental evidence.

*Hence, we have highlighted specific issues we would like you to consider in your review. Please do not speculate, nor respond to any issues about which you believe you do not have expertise.*

**Issue 1: Design of Primedica (2001)**

- Please comment on aspects of the experimental design that would affect the interpretation of the brain morphometry results?
- Were the morphometric methods used appropriate for determining an effect on brain development?
- Is the sampling procedure used in this study for microscopic evaluations appropriate to look for an effect that is mediated through the thyroid hormones or TSH?
- Do the measurements made in this study have inherent sources of variation, and was the inherent variability adequately controlled in the study? How does the variability reported in this study compare with the expected variance?

- Did the techniques used result in adequate homology in the brain sections and did they adequately control for sources of variation in the brain morphometric measurements?

- How does the use of coronal sections affect the ability to compare the results with values reported in the literature?

- How do the linear dimensions of measured brain regions from the control animals in this study compare to literature values?

- How might assessing the morphometry of brain structures in the rat at the ages chosen in this study (PND 10 and PND 22) affect interpretation of whether the findings are biologically significant?

**Issue 2: Biological Significance of Results**

- Is there a 'pattern' to the observed changes that can be interpreted as a consistent effect on brain development, based on the results from the Primedica (2001) study and the Argus (1998) neurobehavioral study?

- There are several significant effects in males at the 1 mg/kg/d dose that are not present at the higher dose (e.g., left striatum and right CA1). Also, there are several parameters that are affected by dose, but that show a similar magnitude of effect over the very large dose (e.g., cerebellum) range in this study. Is there an explanation for these observations of more regions with statistically significantly different measures at the lower doses than the high dose?

- Is there a known or likely explanation for the apparent difference in direction of the change in brain morphometric measurements in males and females (e.g., increases in males, decreases in females)?

- Are the statistically significant changes in linear dimensions observed in this study considered biologically significant? If not, what degree and/or direction of change (i.e., 10% increase, 20% decrease etc.) would be considered biologically significant?

**Issue 3: Biological Plausibility of Results (consistency with mechanism of action)**

- Are the changes observed in linear dimensions of brain regions consistent with changes in the thyroid hormone or thyroid histopathology observed in the same groups of animals?

- Do the results reported for the structures measured correspond to effects observed in published studies of severe hypothyroidism?

- Does the pattern of statistically significant changes observed in the brains correspond to the pattern that would be expected if these changes were being mediated by alterations in thyroid hormone levels?

**Issue 4: Relationship with Behavioral/Functional Effects**

- Are the statistically significant changes observed in linear dimensions of brain regions in pups likely to be associated with behavioral/functional effects in adult animals?

- Have the appropriate behavioral endpoints been examined in order to evaluate an effect of perchlorate exposure on behavior?

**Issue 5: Relevance to Humans**

- Can you comment on whether the brain effects observed in rats would be expected to be observed in humans as well? If changes in linear dimensions of brain regions were to be observed in humans, would these changes result in a functional or behavioral deficit?

- In particular, how might species differences in brain morphometry and development affect the appropriateness of the rat as a model for human neurodevelopment?

## Materials Included in Package

The focus of this review is the Primedica (2001) study. However, we have included materials from other studies in the package in order provide enough background to familiarize you with the issues associated with perchlorate toxicity. Please use it to the extent that you find it useful to address the specific questions asked, but do not feel that you must closely examine everything provided in this package. If you require additional information that has not been included in this package, it will be provided to you; please contact Joan Dollarhide at *TERA*. Examples of information available include the individual animal data and other detailed study results from the Primedica, (2001) and the Argus (1998) studies, reports from earlier toxicity studies, and perchlorate studies available from the general literature.

## Attachment A: The Primedica (2001) Report.

The final report for this study is currently in preparation. Attachment A contains a draft final report as well as additional data tables, the neuropathology report for both PND 10 and PND 22 pups, and reports on serum hormone analyses. All of this material will be included as appendices in the final study report.

- A-1: Draft final report. This draft is relatively complete and contains summary data on thyroid hormones and histology, brain histology and morphometry, and general study design and results.
- A-2: Draft final neuropathology report for PND 10 pups. This report was prepared by Consultants in Veterinary Pathology and contains a detailed description of the brain histology and morphometric methods.
- A-3: Draft final neuropathology report for PND 22 pups. This report was prepared by Consultants in Veterinary Pathology and contains a detailed description of the brain histology and morphometric methods.
- A-4: Photographs of Brain Sections. This attachment provides thumbnail photos of each brain section in the study, and is provided to allow reviewers to assess variability and homology of sections.
- A-5: Dose-response Curves for Brain Morphometry Data. This attachment provides plots of the dose-response curves for each brain region that had at least one statistically significantly increased or decreased measurement. Note, the curves also present the results of regression analyses conducted by TERA, however, the primary purpose of including these curves is to help you visualize the dose-response.

A-6: Serum hormone analyses for gestation day 21 dams and pooled litters.

A-7: Serum hormone analyses for PND 10 dams and pooled litters, and pups culled at PND 5.

A-8: Serum hormone analyses for PND 22 dams and litters pooled by sex, and pups culled at PND 5.

**Attachment B: The Argus 1998 Neurobehavioral Study Report**

This study has been included to provide background on perchlorate neurotoxicity and to assist you in answering specific questions regarding the comparison of the results of the two studies.

B-1: Final report of the Argus (1998) study. The third page of Attachment B-1 (page 20 from the report) shows a schematic of the study design. Page 45 of the study shows the protocol for selection of pups for various endpoints. Pages 46-49 describe the behavioral test conducted. The Results section starts on page 55.

B-2: Serum thyroid hormone measurements for PND5 culled pups and PND10 dams. These are the only thyroid hormone measurements available for this study.

B-3: Thyroid Histopathology from Argus (1998). This attachment represents a reanalysis of the thyroid histopathology; based on criteria developed by a Pathology Working Group and as reported by an EPA pathologist in May, 2001. A major recommendation of the peer review group was to convene a PWG to develop consistent criteria for evaluating thyroid pathology and to re-evaluate all of the earlier studies to get a consistent pathology evaluation. These tables should be considered the definitive data from the Argus, 1998, study and they supplant the histopathology results described in the final report (B-1).

B-4: Memo from an EPA neurotoxicologist. In the original protocol, neurohistology was done only on the control and high dose group. Because a statistically significant effect was seen in the corpus callosum, the next lower dose group was evaluated. This memo (only pages 1-4 of 32 are included) shows summary figures for the brain measurements that showed any statistically significant differences.

B-5: Neurohistology and morphometrics report on the PND12 pups. It contains detailed description of the brain histology and morphometry methods and the results from the control and high dose groups.

B-6: Neurohistology and morphometrics report on the PND82-85 pups. It contains detailed description of the brain histology and morphometry methods and the results from the control and high dose groups.

B-7: Summary data for the Passive Avoidance, Watermaze, and Auditory startle response tests in Argus (1998)

B-8: Summary data from the motor activity testing in Argus (1998). These contain data from measurements of number of movements and time spent in movement for male and female pups at PND 14, 18, 22, and 59.

**Attachment C: Repeat of Motor Activity Study (Bekkedal, 2001)**

Attachment C contains the proposed protocol for this study. The study was performed in accordance with this protocol except that there was a proposal to collect data on rat pup play activity that was not done. The study PI has informed us that repeated measures ANOVA found no difference in activity between the treatment groups. However, the analysis of this data by the U.S. EPA has not been completed and it is possible that other analyses may come to different conclusions.

# Appendix B: Curricula Vita for Reviewers

VICTOR H. DENENBERG

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**Professional Experience**

The University of Connecticut
Professor Emeritus, 2000-
Professor of Biobehavioral Sciences and Psychology, 1969-2000

Purdue University
Assistant Professor to Professor of Psychology, 1954-1969

Human Resources Research Office
Research Associate at HRU Number 1, Fort Knox, Kentucky, 1952-1954

The Jackson Laboratory, Bar Harbor, Maine
Visiting Investigator, Summers, 1955-1962, 1966, 1968-1970, 1972, 1982, 1984, 1986-

Cambridge University, England; Sabbatical leave at Subdepartment of Animal Behaviour, 1963-1964

**Honors or Distinctions**

NIH Special Fellowship, 1963-1964

Annual Research Award, Sigma Xi, Purdue Chapter, 1965

Behavioral Sciences Training Committee, NIGMS, NIH, 1967-1971

Committee on Brain Sciences of the NRC-NAS, 1967-1973

APA Committee on Precautions and Standards in Animal Experimentation, 1967-1970 (Chairman, 1969-1970)

President, International Society of Developmental Psychobiology, 1970

American Association for the Advancement of Science, Committeeman-at-Large of Psychology Section and member, AAAS Council, 1971-1974

Brain Sciences Subcommittee IV: Importance of Early Experiences in Child Development. Assembly of Life Sciences, NRC-NAS, 1973-1978

Division of Comparative and Physiological Psychology, APA; member of Membership Committee, 1970-1973; member of Executive Committee, 1980-1983

Member, Rating Committee to review all doctoral programs in Psychology in the State of New York, 1985-1989

Member, Board of Scientific Affairs, American Psychological Association,1989 -1992

Panel member for the evaluation of the Howard Hughes Medical Institute Fellowship Applications, National Research Council, 1989 - 1992; 1998-1999

Invited address at celebration of the 100th anniversary of the founding of the Pavlov Institute, Leningrad, Russia, USSR, 16-19 April 1991.

Consulting Editor, Journal of Comparative Psychology, 1991-1993

Associate Editor, Psychobiology, Behavioral and Brain Sciences, 1982--

Liason between Science Directorate, APA, and the Society for Neuroscience, 1993 -1998.

Special Review Consultant, NIDA, Drug Abuse Biomedical Research Review Committee, 1990-1994, 1996

Special Review Consultant, NICHD, 1997

Listed in Who's Who in America

Fellow, American Association for the Advancement of Science, American Psychological Association, Animal Behaviour Society

**Major Research Interests**

Animal models of learning disability
Brain laterality and early experiences
Effects of early experiences
Brain-behavior relationships
Sex steroids and behavior
Genes and behavior

## PUBLICATIONS OF VICTOR H. DENENBERG

**1950**

### Research

Ross, S., Smith, W., and Denenberg, V. H. A preliminary study of individual and group hoarding in the white rat. J. Genet. Psychol., 1950, 77, 123-127.

**1952**

### Research

Denenberg, V. H. Hoarding in the white rat under isolation and group conditions. J. Comp. Physiol. Psychol., 1952, 45, 497-503.

**1953**

### Methodology/Apparatus

Denenberg, V. H. A simplified method of measuring kinesthetic reaction- times. Amer. J. Psychol., 1953, 66, 309-311.

**1954**

### Research

Denenberg, V. H. The training effectiveness of a tank hull trainer. U. S. Dept. Army, HumRRO, Tech. Rep. 3, 1954.

Denenberg, V. H. The training effectiveness of the track and suspension system trainer, Device 29-FA-61. Human Res. Unit Nr. 1, Ft. Knox, KY, Information Rep., 1954.

Denenberg, V. H., and Levy, L. Relationships between firing scores for the M1 rifle, carbine, and light machine gun. Human Res. Unit Nr. 1, Ft. Knox, KY, Tech. Rep., 1954.

Denenberg, V. H., and McGuigan, F. J. Evaluation of a special live-fire trigger-squeeze exercise. U. S. Dept. Army, HumRRO, Tech. Rep. 6, 1954.

McGuigan, F. J., and Denenberg, V. H. Accuracy of M1 rifle scores obtained on the known-distance range. U. S. Dept. Army, HumRRO, Res. Mem. 4, 1954.

Tallarico, R. B., Montague, W., and Denenberg, V. H. The map-using proficiency of basic trainees. U. S. Dept. Army, HumRRO, Tech. Rep. 11, 1954.

### Statistics

Denenberg, V. H. Remark on "A qualification in the use of analysis of variance." Psychol. Bull., 1954, 51, 169-171.

## 1956

### Research

Ross, S., Denenberg, V. H., and Chambers, R. M. Development of high level science talent. The Science Teacher, 1956, 23, 279-281, 301, 303.

Ross, S., Denenberg, V. H., Sawin, P. B., and Meyer, P. Changes in nest building behaviour in multiparous rabbits. Brit. J. Anim. Behav., 1956, 4, 69-74.

## 1957

### Research

Denenberg, V. H., and Naylor, J. C. The effects of early food deprivation upon adult learning. Psychol. Rec., 1957, 7, 75-77.

### Theory and/or Review

Ross, S., Ginsburg, B., and Denenberg, V. H. The use of the split-litter technique in psychological research. Psychol. Bull., 1957, 54, 145-151.

## 1958

### Research

Denenberg, V. H. Effects of age and early experience upon conditioning in the C56Bl/10 mouse. J. Psychol., 1958, 46, 211-226.

Denenberg, V. H., King, J. A., and Ehrenfeld, D. W. Effects of testosterone proprionate on the avoidance learning of male C57BL/6 mice. Physiol. Zool., 1958, 31, 244-247.

Denenberg, V. H., and Kline. N. J. The relationship between age and avoidance conditioning in the hooded rat. J. Comp. Physiol. Psychol., 1958, 51, 488-491.

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Denenberg, V. H., Sawin, P. B., Frommer, G. P., and Ross, S. Genetic, physiological and behavioral background of reproduction in the rabbit: IV. An analysis of maternal behavior at successive parturitions. Behaviour, 1958, 13, 131-142.

**1959**

**Research**

Denenberg, V. H. Interactive effects of infantile and adult shock levels upon learning. Psychol. Rep., 1959, 5, 357-364.

Denenberg, V. H. Learning differences in two separated lines of mice. Science, 1959, 130, 451-452.

Denenberg, V. H., and Bell, R. W. Relationships between social reactions and avoidance conditioning. Physiol. Zool., 1959, 32, 51-56.

Denenberg, V. H., and Karas, G. G. Effects of differential infantile handling upon weight gain and mortality in the rat and mouse. Science, 1959, 130, 629-630.

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Ross, S., Chambers, R. M., and Denenberg, V. H. Value profiles of biological science research apprentices. Amer. Inst. Biol. Sci. Bull., 1959, 9, 16-19.

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**1960**

**Research**

Denenberg, V. H. A test of the critical period hypothesis and a further study of the relationship between age and conditioning in the C57Bl/10 mouse. J. Genet. Psychol., 1960, 97, 379-384.

Denenberg, V. H. The relationship between a measure of kinesthesis and two indices of adjustment. J. Gen. Psychol., 1960, 62, 43-52.

Denenberg, V. H., and Bell, R. W. Critical periods for the effects of infantile experience on adult learning. Science, 1960, 131, 227-228.

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# CURRICULUM VITAE

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1978-79 NIMH Postdoctoral Fellow, Dept. of Psychology, University of Illinois, Champaign

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Honors:
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BRSG, 1980; 1981, Indiana University
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BRSG, 1986, 1989, University of Illinois
Research Board, 1986, 1989, 1997 University of Illinois (Both 1986 and 1997 were Arnold O. Beckman awards)

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NIH, "Effects of experience and gender on the hippocampus", 9-81 to 11-84.

John D. & Catharine T. MacArthur Foundation, "Effects of experience and gender on the hippocampus and behavior", 9-81 to 9-85.

NIMH, "Testosterone and cortical development", 8-86 to 2-88.

NSF, "Gender differences in the anatomy of non-reproductive brain areas" plus R.E.U. Supplement, 3-86 to 2-89.

NSF, "Gender differences in anatomy of non-reproductive brain areas", 8-89 to 1-93.

Host to NSF Visiting Professorship for Women grant to Dr. Celia Moore, "Maternal effects on sexually dimorphic neural development", 9-89 to 8-90.

NSF, Subcontract to "Reproductive consequences of maternal stimulation", Celia Moore PI, 5-92 to 8-92.

NSF, "Gender differences in anatomy of non-reproductive brain areas", 8-93 to 2-97.

The Autism Research Foundation (with Black and Greenough), "Quantitative correlates of cognitive dysfunction in autism".

NSF, "Sex differences in anatomy of non-reproductive brain areas", 9-97 to 2-01.

NIA, "Hormonal interaction with aging brain", 3-00 to 4-01

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Physiological basis for learning and memory
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Seminars on the hippocampus and on sex differences
Team taught Behavioral Science for medical students
Seminar on the history of ideas in the biology of behavior

Publications:

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DEPARTMENT OF NEUROBIOLOGY FACULTY

# Pat Levitt, Ph.D.

## Lab Research

Dr. Levitt's laboratory investigates the molecular and cellular mechanisms that regulate the assembly of brain circuitry during development, focusing on the cerebral cortex and regions of the limbic system. The laboratory addresses long-standing issues, such as the control of cell fate, molecular mechanisms of axon-target recognition and cell-cell interactions that are required for growth, differentiation and survival of neurons. They analyze growth factors that are involved in controlling cell phenotype, including a focus on the EGF/heregulin family of ligands and related receptors. Mechanisms of axon targeting are explored through examination of the regulation of cell surface adhesive proteins (CAMs). Cloning and isolation of novel genes, expressed on functionally-related neurons, allow us to perturb specific aspects of development. Technical approaches include gene cloning, protein biochemistry, cell and explant culture, brain transplants, and structural analysis of developmental defects induced through molecular genetic and direct experimental manipulations.

They also investigate the role of neurotransmitters in controlling differentiation of individual neurons, and the response of developing neurons to injury. The effects of in utero exposure to drugs of abuse, which modify neurotransmitter and receptor activities, are examined from basic developmental and cellular perspectives. Studies following brain injury in the developing brain include immunochemical assays to understand the molecular signaling that controls neuroimmune interactions. Future studies in the laboratory will continue to focus upon fundamental issues of developmental neurobiology, with the adaptation of anatomical, biochemical, cellular and molecular methods.

## Rotation Opportunities

Circuit formation in the developing brain requires differential expression of genes that mediate axon pathfinding, targeting and synapse formation. In the cerebral cortex, this involves formation of connections that are likely to be important in neuropsychiatric disorders with a developmental etiology. There are several projects that relate to this central laboratory theme:

1) We use single cell PCR to establish cDNA libraries for screening differential gene expression in identified populations of pyramidal and interneurons in the frontal cortex.

2) The development of functional cortical areas is being investigated in model culture systems in which the actions of growth factor and receptor signaling are tested on isolated progenitor cells.

3) Growth of early axon pathways involved in emotion, memory and learning in rats and in normal and knockout mice are being examined using dye labeling and virus tracers.

4) We are injecting very early embryos with reagents to perturb cortical development, using ultrasound imaging and 5) we are examining developing dopamine systems and cortex in a rabbit animal model of prenatal cocaine exposure.

**Techniques**

gene cloning, sequencing, PCR, library construction and related techniques
gene knockout and transgenic mice production and analysis
fetal surgery
neuroanatomical circuit analysis using tracing methods
in situ hybridization and immunocytochemistry
computer morphometry to assay neuronal growth and differentiation

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## Representative Publications

Levitt, P., Harvey, J.A., Friedman, E., Simansky, K. and Murphy, E.H. 1997. New Evidence for neurotransmitter influences on brain development. Trends in Neurosci. 20:269-274.

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Gao, P.-P., Yue, Y., Zhang, J.-H., Cerretti, D.P., Levitt, P. and Zhou, R. Regulation of thalamic neurite outgrowth by the Eph ligand ephrin-A-5: Implications in the development of thalamocortical projections. Proc. Natl. Acad. Sci. USA 95: 5329-5334, 1998.

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## Contact

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*Biographical sketch of faculty*

# Pat R. Levitt

## Education/Training

| | | | |
|---|---|---|---|
| 1975 | University of Chicago, Chicago | Biological Sciences | B.A. |
| 1978 | University of California, San Diego | Neurosciences | Ph.D. |

## Research Professional Experience

| | | |
|---|---|---|
| 1975-78 | Predoctoral Fellow | Department of Neurosciences, University of California San Diego |
| 1978 | Research Associate | Department of Physiological & Pharmacological Sciences, Univ. of Chicago, Chicago, IL. A. Heller, B. Garber, (6 months) |
| 1979-81 | Postdoctoral Fellow | Section of Neuroanatomy, Yale Univ. School of Medicine, New Haven, CT. P. Rakic sponsor |
| 1981-82 | Assistant Professor | Section of Neuroanatomy, Yale Univ. School of Medicine, New Haven, CT |
| 1982-86 | Assistant Professor | Department of Anatomy, The Medical College of Pennsylvania, Philadelphia, PA |
| 1986-89 | Associate Professor | Department of Anatomy, The Medical College of Pennsylvania, Philadelphia, PA |
| 1989-93 | Professor | Department of Anatomy & Neurobiology, The Medical College of Pennsylvania, Philadelphia, PA |
| 1993-96 | Professor | Department of Neuroscience and Cell Biology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ |
| 1996 - | Professor | Chariman, Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA |
| 1996- | Co-Director | Center for Neuroscience, University of Pittsburgh, School of Medicine, Pittsburgh, PA |

## Extramural:

| | |
|---|---|
| 1986-88 | Regular Panel Member, NSF Developmental Neurobiology Study Section |
| 1987-90 | Course Co-Director Cold Spring Harbor Laboratory Summer Course: Molecular Probes of the Nervous System. |
| 1990-94 | Regular Panel Member, Neuro B1 Study Section, NIH |
| 1990-97 | Associate Editor, Joural of Neuroscience |
| 1997-present | Reviewing Editor, Journal of Neuroscience |

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|---|---|
| 1991-present | Associate Editor, Perspectives on Developmental Neurobiology |
| 1997-present | Associate Editor, Neuron |
| 1995-present | Advisory Board, Biological Psychiatry |
| 1994-present | Society for Neuroscience, Program Committee |
| 1996-97 | Society for Neuroscience, Council |

## Awards

| | |
|---|---|
| 1989-99 | NIMH, MERIT Award |
| 1995-96 | NARSAD Established Investigator Award |
| 1988-89 | Scottish Rite Schizophrenia Foundation Investigator Award |
| 1985-88 | Klingenstein Foundation Fellowship July |
| 1983-85 | National Downs Syndrome Society Scholar Award |
| 1982-85 | Alfred P. Sloan Research Fellow |
| 1979-81 | National Institutes of Health NINCDS Postdoctoral Fellowship |

## Selected of Publications*(41 of 85)*


1. Levitt, P. A monoclonal antibody to limbic system neurons. Science 223: 299-301, 1984.

2. Horton, H.L. and Levitt, P. A unique membrane protein is expressed on early developing limbic system axons and cortical targets. J. Neurosci. 8: 4653-4661, 1988.

3. Keller, F., Rimvall, K., Barbe, M.F. and Levitt, P. A membrane glycoprotein associated with the limbic system mediates the formation of the septo-hippocampal pathway in vitro. Neuron 3:551-561, 1989.

4. Zacco, A., Cooper, V., Chantler, P.D., Hyland-Fisher, S., Horton, H.L. and Levitt, P. Isolation, biochemical characterization and ultrastructural analysis of the limbic system-associated membrane protein (LAMP), a protein expressed by neurons comprising functional neural circutis. J. Neurosci. 10: 73-90, 1990.

5. Pennypacker, K.P., Fischer, I. and Levitt, P. Early in vitro genesis and differentiation of axons and dendrites by hippocampal neurons analyzed quantitatively, with neurofilament-H and microtubule-associated protein 2 antibodies. Exp. Neurol., 111: 25-35, 1991.

6. Chesselet, M.-F., Gonzalesz, C. and Levitt, P. Heterogeneous distribution of the limbic system-associated membrane protein (LAMP) in the caudate nucleus and substantia nigra of the cat. Neurosci., 40: 725-733, 1990.

7. Barbe, M.F. and Levitt, P. The early committment of fetal neurons to limbic cortex. J. Neurosci. 11: 519-533, 1991.

8. Rinaman, L. and Levitt, P. Access to gastric tissue promotes the survival of axotomized neurons in the dorsal motor nucleus of the vagus in neonatal rats. J. Comp. Neurol., 313: 213-226, 1991

9. Milligan, C.E., Levitt, P. and Cunningham, T.J. Brain macrophages and microglia respond differently to lesions of the developing and adult visual system. J. Comp. Neurol. 314: 136-146, 1991.

10. Barbe, M.F. and Levitt, P. Attraction of specific thalamic input by cerebral grafts depends on the molecular identity of the implant. Proc. Nat'l. Acad. Sci. (USA) 89: 3706-3710, 1992.

11. Miller, M., Bower, E., Levitt, P., Li, D. and Chantler, P.D. Myosin II distribution in neurons is consistent with a role in growth cone motility but not synaptic vesicle mobilization. Neuron 8: 25-44, 1992.

12. Qian, J. and Levitt, P. Target-derived astroglia regulates axonal growth in a region-specific manner in vitro. Dev. Biol., 149:278-294, 1992.

13. Silver, J., Edwards, M., Levitt, P. Immunocytochemical demonstration of early appearing astroglial structures that form boundaries and pathways along axon tracts in the fetal brain. J. Comp. Neurol., 328: 415-436, 1993.

14. Prouty, S. and Levitt, P. Immunocytochemical analysis of a novel carbohydrate differentiation antigen (CDA-3C2) associated with olfactory and optic systems during embryogenesis in the rat. J. Comp. Neurol. 332: 444-470, 1993.

15. Levitt, P., Ferri, R.T. and Barbe, M.F. Progressive acquisition of cortical phenotypes as a mechanism for specifying the developing cerebral cortex. Perspect. Dev. Neurobiol. 1: 65-74, 1993.

16. Ferri, R.T. and Levitt, P. Cerebral cortical progenitors are fated to produce region-specific neuronal populations. Cerebral Cortex, 3: 187-198, 1993.

17. Hockfield, S., Carlson, S., Evans, C., Levitt, P., Pinter, J. and Silberstein, L. Molecular Probes of the Nervous System. Cold Spring Harbor Press, New York, 1994.

18. Qian, J., Wang, H.-Y., Fischer, I., Friedman, E. and Levitt, P. The involvement of protein kinase C in axonal growth-promoting effect on spinal cord neurons by target-derived astrocytes. J. Neurobiol., 25: 1593-1612, 1994.

19. Barbe, M.F. and Levitt, P. Age-dependent specification of the cortico-cortical connections of cerebral grafts. J. Neurosci. 15: 1819-1834, 1995.

20. Zhukareva, V. and Levitt, P. The limbic system-associated membrane protein

(LAMP) selectively mediates interactions with specific central neuron populations. Development 121: 1161-1172, 1995.

21. Ferri, R.T. and Levitt, P. Regulation of retgional differences in the differentiation of cerebral cortical neurons by EGF family-matrix interactions. Development 121: 1151-1160. 1995.

22. Milligan, C.E., Webster, L., Piros, E.T., Evans, C., Cunningham, T.J. and Levitt, P. Induction of opiate receptor-mediated macrophage chemotactic activity following neonatal brain injury. J. Immunol. 154: 6571-6581, 1995.

23. Pimenta, A., Zhukareva, V., Barbe, M.F., Reinoso, B., Grimley, C., Henzel, W., Fischer, I. and Levitt, P. The limbic system-associated membrane protein is an ig superfamily member that mediates selective neuronal growth and axon targeting. Neuron 14: 1-15, 1995.

24. Eagleson, K.L., Ferri, R.T., Levitt, P. Complementary distribution of collagen type IV and the epidermal growth factor receptor in the embryonic telencephalon. Cereb. Cortex 6: 540-549, 1996.

25. Pimenta, A., Fischer, I. and Levitt, P. Molecular cloning and structural analysis of the human limbic system associated membrane protein (LAMP). Gene 170: 189-195, 1996.

26. Ferri, R.T., Eagelson, K. and Levitt, P. Environmental signals influence a cortical areal phenotype in vitro independent of effects on progenitor cell proliferation. Dev. Biol. 175: 184-190 1996.

27. Smigrodzki, R. and Levitt, Pat. The subunit of soluble guanylyl cyclase is expressed prenatally in the rat brain. Dev. Brain Res. 97: 226-234, 1996.

28. Levitt, P., Eagleson, K.L., Chan, A.V., Ferri, R.T. and Lillien, L. Signaling pathways that regulate specification of neurons in developing cerebral cortex. Dev. Neurosci. 19: 6-8, 1996.

29. Levitt, P., Barbe, M.F. and Eagleson, K.L. Patterning and specification of the cerebral cortex. Ann. Rev. Neurosci. 20: 1-124, 1997.

30. Levitt, P., Harvey, J.A., Eitan Friedman, E., Simansky, F. and Murphy, E.H. New evidence for neurotransmitter influences on brain development. TINS 20: 269-274, 1997.

31. Eagleson, K.L., Lillien, L., Chan, A.V. and Levitt, P. Mechanisms specifying area fate in cortex include cell-cycle-dependent decisions and the capacity of progenitors to express phenotype memory. Development 124: 1623-1630, 1997.

32. Levitt, P., Harvey, John A., Friedman, E. and Murphy E. H. New evidence for neurotransmitter influences on brain development. TINS 20: 269-274-1997. Publications (cont.)

33. Burrows, R.C., Wancio, D., Levitt, P. and Lillien, L. Response diversity and the

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timing of progenitor cell maturation are regulated by developmental changes in EGF-R expression in the cortex. Neuron 19: 251-267, 1997.

34. Murphy, Hazel, E., Itzhak, F., Friedman. E., Grayson, D., Jones, L., Levitt, P., O'Brien-Jenkins, A., Wang, H.-Y. and Wang, X.-H. Cocaine administration in pregnant rabbits alters cortical structure and function in their progeny in the absence of maternal seizures. Exp. Brain Res. 114: 433-441, 1997.

35. Zukareva, V., Chernevskaya, N., Pimenta, A., Nowycky, M. and Levitt, P. Limbic system-associated membrane protein (LAMP) induces neurite outgrowth and intracellular Ca2+ increase in primary fetal neurons. Molec. and Cell. Neurosci. 10: 43-55, 1997.

36. Zhang, J-H., Pimenta, A.F., Levitt, P. and Zhou, R. Dynamic expression suggests multiple roles of the eph family receptor brain-specific (Bsk) during mouse neurogenesis. Molec. Brain Res. 47: 202-214, 1997.

37. Zhang, B., Levitt, P. and Murray, M. Induction of presynaptic re-expression of an adhesion protein in lamina II after dorsal root deafferentation in adult rat spinal cord. Exp. Neurol. 149: 468-472, 1998

38. Gao, P.-P., Yue, Y., Zhang, J.-H., Cerretti, D.P., Levitt, P. and Zhou, R. Regulation of thalamic neurite outgrowth by the Eph ligand ephrin-A-5: Implications in the development of thalamocortical projections. Proc. Nat. Acad. Sci. 95: 5329-5334, 1998.

39. Card, J.P., Levitt, P. and Enquist, L.W. Different patterns of neuronal injection after intracerebral injections of two strains of pseudorabies virus. J. Virol. 72: 4434-4441, 1998.

40. Pimenta, A.F., Tsui, L.-C., Heng, H.H.Q. and Levitt, P. Assignment of the gene encoding the limbic system- associated membrane protein (LAMP) to mouse chromosome 16B5 and human Chromosome 3 q13.2-q21. Genomics 49: 472-474, 1998.

41. Schnabel, C.A., Moticka, J.A., Wang, H., Yang, L., Eagleson, K.L., Chan, J., Pickel, V.M. Levitt, P., Schenn, M.M. and Abate-Shen, C. Localization of Hox A7 to axons during neural development. Dev. Biol. (under review).

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Back to Dr. Levitt's Homepage

## CURRICULUM VITAE

| | |
|---|---|
| NAME: | Harold Leon Schwartz S.S.# 118-24-3885 |
| DATE OF BIRTH: | March 14, 1933 |
| MARITAL STATUS: | Married, three children |
| MILITARY SERVICE: | U.S. Army, 1953-55; Honorable Discharge, 1961 |
| EDUCATION: | Brooklyn College B.S. 1957<br>New York University M.S. 1961<br>New York University Ph.D. 1964 |

APPOINTMENTS:

| | |
|---|---|
| 1957-1964 | Biochemist, Endocrine Section, Department of Medicine, Downstate Medical Center, State University of New York |
| 1964-1966 | Instructor, Department of Medicine, State University of New York |
| 1965-1967 | Postdoctoral Fellow, National Institutes of Health, in the laboratory of Dr. Rosalind Pitt-Rivers, National Institute for Medical Research, Mill Hill, London, England |
| 1967-1976 | Research Biochemist, Endocrine Research Laboratory, Montefiore Hospital and Medical Center, Bronx, New York |
| 1967-1976 | Assistant Professor of Biochemistry, Albert Einstein College of Medicine, Bronx, New York |
| 1976-1992 | Associate Professor of Medicine Minnesota Medical School |
| 1976-1992 | Associate Professor of Cell Biology and Neuroanatomy University of Minnesota College of Medicine |
| 1992-1997 | Professor of Cell Biology and Neuroanatomy University of Minnesota College of Mdicine |
| 1992-1997 | Professor of Medicine University of Minnesota College of Medicine |
| 1997-present | Professor Emeritus, Department of Medicine and the Department of Cell Biology and Neuroanatomy |
| 1997- | Clinical Professor of Medicine University of California at Irvine |

Member-VA Merit Review Committee for Endocrinology, 1995-1999

## PUBLICATIONS


1. Cirprut S, Silverstein JN, Schwartz HL, Feldman EB, Carter AC: Effect of common drugs on urinary excretion of gonadotropins. J Clin Endocrinol Metab 22:535-536, 1962.

2. Silverstein JN, Schwartz HL, Feldman EB, Kydd DM, Carter AC: Correlation of the red blood cell uptake of $I^{131}$-L-triiodothyronine and thyroxine-binding globulin in man. J Clin Endocrinol Metab 22:1002-1006, 1962.

3. Carter AC, Schwartz HL, Kydd DM, Koluglu S: Relation of red blood cells $I^{131}$-L-triiodothyronine binding coefficient and cell maturation. Endocrinology 74:689-693, 1964.

4. Koluglu S, Schwartz HL, Carter AC: Quantitative determination of the thyroxine, triiodothyronine, monoidotyrosine and diiodotyrosine content of dessicated thyroid. Endocrinology 78:231-239, 1966.

5. Schwartz HL, Carter AC, Kydd DM, Gordon AS: Relationship of red blood cell $I^{131}$-L-triiodothyronine binding coefficient and cell maturation II. Effect of cell age and metabolic inhibitors. Endocrinology 80:65-68, 1967.

6. Pitt-Rivers R, Schwartz HL: The thiol groups of thyroglobulin. Biochemical J 105:28c, 1967.

7. Schwartz HL, Carter AC, Singh SP, Kydd DM, Costanzo RR: Relationship of red blood cell $^{131}$I-L-triiodothyronine binding coefficient and cell maturation III. Binding to mature erythrocyte and reticulocyte membranes. Endocrinology 82:569-574, 1968.

8. Pitt-Rivers R, Schwartz HL: Iodoamino acids and related compounds. In <u>Chromatographic and Electrophoretic Techniques</u>. Third edition, I Smith, ed., Heinemann, Ltd., London 1969, pp.224-242.

9. Schwartz HL, Bernstein G, Oppenheimer JH: Effect of phenobarbital administration on the subcellular distribution of $^{125}$I-thyroxine in rat liver: Importance of microsomal binding. Endocrinology 84:270-276, 1969.

10. Oppenheimer JH, Surks MI, Schwartz HL: The metabolic significance of exchangeable cellular thyroxine. Recent Progress in Hormone Research 25:381-422, 1969.

11. Schwartz HL, Kozyreff V, Surks MI, Oppenheimer JH: Increased deiodination of L-thyroxine and L-triiodothyronine by liver microsomes from rats treated with phenobarbital. Nature 221:1262-1263, 1969.

12. Surks MI, Schwartz HL, Oppenheimer JH: Tissue iodoprotein formation during the peripheral metabolism of the thyroid hormones. J Clin Invest 48:2168-2175, 1969.

26. Oppenheimer JH, Schwartz HL, Surks MI: Determination of common parameters of iodothyronine metabolism and distribution in man by noncompartmental analysis. J Clin Endocrinol Metab. 41:319-324, 1975. Erratum: Revised calculations of common parameters of iodothyronine metabolism and distribution by noncompartmental analysis. J Clin Endocrinol Metab 41:1172-1173, 1975.

27. Oppenheimer JH, Schwartz HL, Surks MI, Koerner DH, Dillmann W: Physiological significance of nuclear receptor sites. Proc Int Conf Thyroid Horm Metabolism, Glasgow, Scotland, Academic Press, 1975.

28. Koerner D, Schwartz HL, Surks MI, Oppenheimer JH, Jorgensen EC: Binding of selected iodothyronine analogues to receptor sites of isolated rat hepatic nuclei: high correlation between structural requirements for nuclear binding and biological activity. J Biol Chem 250:6417-6423, 1975.

29. Goslings B, Schwartz HL, Dillmann W, Surks MI, Oppenheimer JH: Comparison of the metabolism and distribution of L-triiodothyronine and triiodothyroacetic acid in the rat: a possible explanation of differential hormonal potency. Endocrinology 98:666-675, 1976.

30. Dillmann W, Schwartz HL, Silva E, Surks MI, Oppenheimer JH: Effect of alpha-amanitin on the induction of hepatic enzymes by triiodothyronine. Proc 7th Int Thyroid Conf, Boston, 1975. Excerpta Medica, Int Congress Series No. 378, Amsterdam, 1976, pp. 326-330.

31. Oppenheimer JH, Schwartz HL, Surks MI, Koerner DH, Dillmann W: Nuclear receptors and initiation of thyroid hormone action. Rec Prog Horm Res 32:529-565, 1976.

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33. Oppenheimer JH, Surks MI, Schwartz HL, Koerner DH, Dillmann W: Evidence in support of a nuclear site for the initiation of thyroid hormone action. In Regulation of Thyroid Function, E Klein and RD Reinwein, eds., FK Schattauer, Stuttgart, 1976, pp. 131-141.

34. Dillmann WH, Schwartz HL, Silva E, Surks MI, Oppenheimer JH: Alpha-amanitin iadministration results in a temporary inhibition of hepatic enzyme induction by triiodothyronine: further evidence favoring a long-lived mediator of thyroid hormone action. Endocrinology 100:1621-1627, 1977.

35. Oppenheimer JH, Silva E, Schwartz HL, Surks MI: Stimulation of hepatic mitochondrial $\alpha$-glycerophosphate dehydrogenase and malic enzyme by L-triiodothyronine: characteristics of the response with specific nuclear thyroid hormone binding sites fully saturated. J Clin Invest 59:517-527, 1977.

36. Silva ES, Astier H, Thakare U, Schwartz HL, Oppenheimer JH: Partial purification of the triiodothyronine receptor from rat liver nuclei: differences in the chromatographic mobility of occupied and unoccupied sites. J Biol Chem 252:6799, 1977.

thyroid hormone and high carbohydrate diet in the induction of lipogenic enzymes in the rat: mechanisms and implications. J Clin Invest 65:1126-1134, 1980.

51. Kaiser FE, Mariash CN, Schwartz HL, Oppenheimer JH: Inhibition of malic enzyme induction of triiodothyronine in the diabetic rat: reversal by fructose feeding. Metabolism 29:767-772, 1980.

52. Oppenheimer JH, Schwartz HL: Factors determining the level of activity of 3,5,3'-triiodothyronine-responsive hepatic enzymes in the starved rat. Endocrinology 107:1460-1468, 1980.

53. Schwartz HL, Lancer SR, Oppenheimer JH: Thyroid hormones influence starvation-induced hepatic protein loss in the rat: possible role of thyroid hormones in the generation of labile protein. Endocrinology 107:1684-1692, 1980.

54. Simat BM, Towle HC, Schwartz HL, Oppenheimer JH: Difference between thyroidectomized and hypophysectomized animals in their hepatic ribonucleic acid response to thyroid hormone. Endocrinology 107:1338-1344, 1980.

55. Bantle JP, Oppenheimer JH, Schwartz HL, Hunninghake DB, Probstfield JL, Hanson RF: TSH response to TRH in euthyroid, hypercholesterolemic patients treated with graded doses of dextrothyroxine. Metabolism 30:63-66, 1981.

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57. Schwartz HL, Oppenheimer JH: Starvation effects on hepatic enzyme response to triiodothyronine and hepatic protein degradation. In The Low T3 Syndrome - Importance of rT3. Serono Symposia, Rome, 1980, pp. 89-100.

58. Towle HC, Mariash CN, Schwartz HL, Oppenheimer JH: Quantitation of rat liver messenger ribonucleic acid for malic enzyme during induction of thyroid hormone. Biochemistry 20:3486-3492, 1981.

59. Jump DB, Seelig S, Schwartz HL, Oppenheimer JH: Association of the thyroid hormone receptor with rat liver chromatin. Biochemistry 20:6781-6789.

60. Forciea MA, Schwartz HL, Towle HC, Mariash CN, Kaiser FE, Oppenheimer JH: Thyroid hormone-carbohydrate interaction in the rat: correlation between age-related reductions in the inducibility of hepatic malic enzyme by triiodo-L-thyronine and a high carbohydrate, fat-free diet. J Clin Invest 67:1739-1747, 1981.

61. Oppenheimer JH, Mariash CN, Towle HC, Schwartz HL, Kaiser FE: Interaction of T3 and carbohydrate in the induction of lipogenic enzymes. Life Sciences 28:1693, 1981.

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63. Oppenheimer JH, Schwartz HL, Mariash CN, Kaiser FE: Evidence for a factor in

transnuclear transport of 3,5,3'-triiodothyronine in isolated hepatocytes. Endocrinology 117:2449-2456, 1985.

76. Freake HC, Mooradian AD, Schwartz HL, Oppenheimer JH: Stereospecific transport of triiodothyronine to cytoplasm and nucleus in GH1cells. Mol Cell Endocrinol 44:25-35, 1986.

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78. Oppenheimer JH, Schwartz HL: Thyroid hormone action at the nuclear level. In Thyroid Hormone Metabolism (G Hennemann, ed.), New York: Marcel Dekker, 1986, pp. 383-415.

79. Mariash CN, Seelig S, Schwartz HL, Oppenheimer JH: Rapid synergistic interaction between thyroid hormone and carbohydrate on mRNA-S14 induction. J Biol Chem 261:9583, 1986.

80. Kinlaw WB, Schwartz HL, Towle HC, Oppenheimer JH: Opposing effects of glucagon and triiodothyronine on the hepatic levels of messenger ribonucleic acid S14 and the dependence of such effects on circadian factors. J Clin Invest 78:1091, 1986.

81. Oppenheimer JH, Schwartz HL, Mariash CN, Jump DB, Kinlaw WB: Current concepts of thyroid hormone action at the molecular level: possible clues in understanding the role of thyroid hormone in brain development. International Symposium Workshop on Iodine Nutrition, Thyroxine and Brain Development, New Delhi, 1987, pp. 138-149.

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85. Mariash CN, Kinlaw WB, Schwartz HL, Freake HC, Oppenheimer JH: Interaction of thyroid hormone and carbohydrates on hepatic gene expression. In Gene Regulation by Steroid Hormones III (AK Roy, JH Clark, eds.), Meadow Brook Conference on the Molecular Mechanism of Steroid Hormone Action. Springer-Verlag, 1987, pp. 273-297.

86. Weirich RT, Schwartz HL, Oppenheimer JH: An analysis of the interrelationship of nuclear and plasma triiodothyronine in the sea lamprey eel, lake trout, and the rat: evolutionary considerations. Endocrinology 120:664-677, 1987.

Thieme Verlag, Stuttgart, 1987.

99. Santos A, Freake HC, Rosenberg ME, Schwartz HL, Oppenheimer JH: Triiodothyronine nuclear binding capacity in rat tissues correlates with a 6.0 kb and not a 2.6 kb messenger ribonucleic acid hybridization signal generated by a human c-erbA probe. Mol. Endocrinol. 2:992-998, 1988.

100. Oppenheimer JH, Freake HC, Santos A, Perez-Castillo AM, Schwartz HL, Kinlaw WB, Mariash CN, Strait K: Lipogenesis, Thermogenesis, and the Induction of mRNA S-14 by Thyroid Hormone. Sternbrook Symposium: Hormones, Thermogenesis and Obesity, University of Wisconsin, Madison, WI, June, 1988.

101. Mariash, CN, Kinlaw WB, Schwartz HL, Freake HC, Oppenheimer JH: Interaction of thyroid hormone and carbohydrates on hepatic gene expression. in Gene Regulation by Steroid Hormones III (AK Roy, JH Clark, eds.), Meadow Brook Conference on the Molecular Mechanism of Steroid Hormone Action. Springer-Verlag, 1987, pp 273-297.

102. Kinlaw, WB, Schwartz, HL, Hamblin, PS, Mariash, CN, Oppenheimer, HL: Triiodothyronine rapidly reverses inhibition of S14 gene transcription by glucagon. Endocrinology 123:2255-2260, 1988.

103. Day R, Gebhard RL, Schwartz HL, Strait K, Duane WC, Stone BG, Oppenheimer JH: Time course of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and mRNA, biliary secretion and hepatic cholesterol content in methimazole-treated hypothyroid and hypophysectomized rats following triiodothyronine administration: possible linkage of cholesterol synthesis to biliary secretion. Endocrinology 125:459-468, 1989.

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106. Freake HC, Schwartz HL, Oppenheimer JH: The regulation of lipogenesis by thyroid hormone and its contribution to thermogenesis. Endocrinol 125:2868-2874, 1989.

107. Scheuler PA, Schwartz HL, Strait KA, Oppenheimer JH: Binding of 3,5,3'-triiodothyronine (T3) and its analogues to the in vitro translational products of c-erbA protooncogenes: differences in the affinity of the α- and β- forms for the acetic acid analogue and failure of human testis and kidney α-2 products to bind T3. Mol Endocrinol 4:227-234, 1990.

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enzyme by thyroid hormone in the neonatal rat. Biochem Biophys Res Comm 222: 287-291, 1996

124. Ercan-Fang, S, Schwartz, HL and Oppenheimer, JH. Isoform-specific 3,5,3'-triiodothyronine receptor binding capacity and messenger ribonucleic acid content in rat adenohypophysis: effect of thyroidal state and comparison with extrapituitary tissues. Endocrinology 137: 3228-3233, 1996

125 Oppenheimer, JH, Schwartz, HL and Strait, KA The Molecular Basis of Thyroid Hormone Actions in Werner and Ingbar's The Thyroid, Braverman, LE and Utiger, RD, eds. 7th Edition, pp. 162-184, 1996

126. Strait, KA, Carlson, DJ, Schwartz, HL and Oppenheimer, JH, Transient stimulation of myelin basic protein gene expression in differentiating cultured oligodendrocytes: a model for 3,5,3'-triiodothyronine-induced brain development. Endocrinology 138: 635-641, 1997

127. Oppenheimer, JH and Schwartz, HL Molecular Basis of Thyroid Hormone-Dependent Brain Development. Endocrine Reviews 18:462-475, 1997

128. Schwartz, HL, Ross, ME and Oppenheimer, JH, Lack of effect of thyroid hormone on late fetal rat brain development. Endocrinology 138:3119-3124, 1997

129. Anderson, GW, Hagen, SG, Larson, RJ, Strait, KA, Schwartz, HL, Mariash, CN, and Oppenheimer, JH, Purkinje Cell Protein-2 cis-elements mediate repression of T3-dependent transcriptional activation. Mol. Cell Endo 131:79-87 1997

130. Sandhofer, CR, Schwartz, HL, Mariash, CN, Forrest, D and Oppenheimer, JH Beta receptor isoforms are not essential for thyroid hormone-dependent acceleration of Pcp-2 and myelin basic protein gene expression in the developing brains of neonatal rats. Mol. Cell Endo. 137: 109-115 1998

131. Anderson, GW, Larson, RJ, Oas, DR, Sandhofer, CR, Schwartz, HL, Mariash, CN, and Oppenheimer, JH COUP-TF modulates expression of the Purkinje cell protein-2 gene: a potential role for COUP-TF in repressing premature thyroid hormone action in the developing brain. J. Biol. Chem. 273:16391-16399, 1998

132. Ercan-Fang, S, Schwartz, HL, Mariash, CN and Oppenheimer, JH Quantitative assessment of pituitary resistance to thyroid hormone from plots of the logarithm of thyrotropin versus serum free thyroxine index. J. Clin. Endocrinol. Metab 85:2299-2303, 2000

*CURRICULUM VITAE*

## Douglas L. Wahlsten

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Edmonton, Alberta, Canada T6G 2E9
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***Birthdate***: October 13, 1943

***Citizenship***: Canadian

***Education, Honours and Positions:***

| | |
|---|---|
| 1965: | B.Sc. Physics, Magna Cum Laude, Alma College, Alma, Michigan. |
| 1965 - 1966: | Graduate student, Department of Psychology, Yale University. |
| 1966 - 1968: | Ph.D. student, Regent's Fellow, University of California, Irvine. |
| 1968 - 1969: | N.I.M.H. Postdoctoral Fellow, Institute for Behavioral Genetics, University of Colorado, Boulder. |
| 1969: | Ph.D. Psychology, University of California, Irvine. |
| 1969 - 1974: | Assistant Professor of Psychology, University of Waterloo, Waterloo, Ontario. |
| Fall 1971: | Visiting Assistant Professor of Psychobiology, University of California, Irvine. |
| 1973: | Steacie Fellowship (NSERC) nominee from University of Waterloo. |
| 1974 - 1982: | Associate Professor of Psychology, University of Waterloo. |
| 1982 - 1989: | Professor of Psychology, University of Waterloo. |
| 1989 - pres: | Professor of Psychology, University of Alberta. |
| 1990 - pres: | Adjunct Professor, Division of Neuroscience, University of Alberta. |
| 1996: | Biography in *Canadian Who's Who* |
| 1999: | Professor Emeritus, University of Alberta |

***Professional Affiliations:***

American Association for the Advancement of Science
International Behavioural and Neural Genetics Society; president, 2000-2001

***Current Research Support***

Natural Sciences and Engineering Research Council of Canada; 1998 to 2002, $31,000/year
National Institutes of Health (USA); 2000 to 2003, $147,000(USD)/year

***Publications***:

**IN PRESS**

Wahlsten, D. Standardizing tests of mouse behavior: reasons, recommendations and reality. Physiology and Behavior, in press.

Sokolowski, M., and Wahlsten, D. Gene-environment interaction. In H. Chin and S. O. Moldin (eds.), Methods in Neurogenetics. Boca Raton, FL: CRC Press, in press.

Wahlsten, D. Genetics and the development of brain and behavior. In J. Valsiner and K. J. Connolly (eds.), Handbook of Developmental Psychology, in press.

Wahlsten, D. The theory of biological intelligence: history and a critical appraisal. In R. Sternberg and E. Gigorenko (eds.), The General Factor of Intelligence: How General Is It?. Mahwah, NJ: Erlbaum, in press.

**2000**

Wahlsten, D. Behavior genetics. Encyclopedia of Psychology. New York: Oxford University Press (sponsored by the American Psychological Association).

Wahlsten, D. (2000). Analysis of variance in the service of interactionism. Human Development, 43: 46-50.

**1999**

Crabbe, J.C., Wahlsten, D., and Dudek, B.C. (1999). Genetics of mouse behavior: Interactions with lab environment. Science, 284: 1670-1672.

Wahlsten, D., Crabbe, J, and Dudek, B. (1999) Testing the genetics of behavior in mice. Response. Science, 285: 2069-2070.

Wahlsten, D. (1999) Single-gene influences on brain and behavior. Annual Review of Psychology, 50: 599-624.

Bishop, K.M, and Wahlsten, D. (1999) Sex and species differences in mouse and rat forebrain commissures depend on the method of adjusting for brain size. Brain Research, 815: 358-366.

Wahlsten, D. (1999) Experimental design and statistical inference. In W.E. Crusio and R. T. Gerlai (eds.), Molecular-genetic Techniques for Behavioral Neuroscience. Amsterdam: Elsevier, pp. 40-57.

Wahlsten, D. (1999). Planning genetic experiments: power and sample size. In B Jones and P. Mormède (Eds.),. Neurobehavioral Genetics. Methods and Applications. New York: CRC Press,

pp. 31-42.

Carlier, M., Roubertoux, P.L., and Wahlsten, D. (1999). Maternal effects in behavior genetic analysis. In B Jones and P. Mormède (Eds.), Neurobehavioral Genetics. Methods and Applications. New York: CRC Press, pp. 187-197.

Wahlsten, D. (1999) The eugenics of John. M. MacEachran warrant revocation of honours. History of Psychology and Philosophy Bulletin, 10: 22-25.

**1998**

Gottlieb, G., Wahlsten, D., and Lickliter, R. The significance of biology for human development: a developmental psychobiological systems view. In R. Lerner (Ed.), Handbook of Child Psychology, Vol. 1, Theory. New York: Wiley, 1998, pp. 233-273.

Wahlsten, D., and Bishop, K.M. Effect sizes and meta-analysis indicate no sex differences in the corpus callosum. Behavioral and Brain Sciences, 1998, 21, 338-339.

Ozaki, H.S., and Wahlsten, D. Timing and origin of the first cortical axons to project through the corpus callosum and the subsequent emergence of callosal projection cells in the mouse. Journal of Comparative Neurology, 1998, 400, 197-206.

Wahlsten, D. (1998). Origins of genetic determinism in medieval creationism. Race, Gender & Class, 5: 90-107.

**1997**

Bishop, K., and Wahlsten, D. Sex differences in the human corpus callosum: Myth or reality? Neuroscience and Biobehavioral Reviews, 1997, 21, 581-601.

Livy, D. J., and Wahlsten, D. Formation of the hippocampal commissure in normal and acallosal mouse embryos. Hippocampus, 1997, 7, 2-14.

Wahlsten, D. Leilani Muir versus the Philosopher King: eugenics on trial in Alberta. Genetica, 1997. 99. 185-198.

Wahlsten, D. The malleability of intelligence is not constrained by heritability. In B. Devlin, et al. (Eds.), Intelligence, Genes & Success. New York: Copernicus (Springer Verlag),1997, pp. 71-87.

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Livy, D.J., Schalomon, P.M., Roy, M., Zacharias, M.C., Pimenta, J., Lent, R., and Wahlsten, D.

Increased axon number in the anterior commissure of mice lacking a corpus callosum. Experimental Neurology, 1997, 146, 491-501.

Wahlsten, D., Bishop, K., and Kruyer, A. Calibration of computer-monitored running wheels with adjustable drag. Behavior Research Methods, Instruments, & Computers, 1997, 29, 280-285.

**1996**

Wahlsten, D. Evaluating genetic models of cognitive evolution. Behavioural Processes, 1996, 35, 183-194.

Wahlsten, D. Advances in genetic analysis of IQ await a better understanding of environment. In D. K. Detterman (Ed.), Current Topics in Human Intelligence, Vol. 5. Norwood, NJ: Ablex Publishing, 1996, pp. 185-190.

Bishop, K., Kruyer, A., and Wahlsten, D. Agenesis of the corpus callosum and voluntary wheel running in mice. Psychobiology, 1996, 24, 187-194.

**1995**

Schalomon, P. M, and Wahlsten, D. A precision surgical approach for complete or partial callostomy in the mouse. Physiology and Behavior, 1995, 57, 1199-1203.

Wahlsten, D. Review of "Race, Evolution and Behavior" by J. P. Rushton. Canadian Journal of Sociology, 1995, 20, 129-133.

Wahlsten, D. The genetic kaleidoscope of vision. Behavioral and Brain Sciences, 1995, 18,490-492.

Wahlsten, D. Sensitivity of the t test to different models of interaction. Cahiers de Psychologie Cognitive, 1995, 14, 205-213.

Wahlsten, D. Increasing the raw intelligence of a nation is constrained by ignorance, not its citizen's genes. Alberta Journal of Educational Research, 1995, 41, 257-264.

**1994**

Wahlsten, D., and Ozaki, H.S. Defects of the fetal forebrain in acallosal mice. In: M. Lassonde & M. Jeeves (Eds.), Callosal Agenesis, New York: Plenum Press, 1994. pp. 125-133.

Wahlsten, D., and Bulman-Fleming, B. Retarded growth of the medial septum: A major gene effect in acallosal mice. Developmental Brain Research, 1994, 77, 203-214.

Wahlsten, D. The intelligence of heritability. Canadian Psychology, 1994, 35, 244-258.

Wahlsten, D. Nascent doubts may presage conceptual clarity. Canadian Psychology, 1994, 35, 265-267 (reply to commentary by M.K. Surbey).

Wahlsten, D. Probability and the understanding of individual differences. In J. Brzezinski (Ed.), Probability in theory-building. Amsterdam: RODOPI, 1994, 39, pp. 47-60.

Wahlsten, D., and Schalomon, P.M. A new hybrid mouse model for agenesis of the corpus callosum. Behavioral Brain Research, 1994, 64, 111-117.

**1993**

Wahlsten, D. Sociobiology flops again. Behavioral and Brain Sciences 1993, 16, 310.

Wahlsten, D. Sample size requirements for the Capron and Duyme balanced fostering study of I.Q. International Journal of Psychology, 1993, 28, 509-516.

Ozaki, H.S., and Wahlsten, D. Cortical axon trajectories and growth cone morphologies in fetuses of acallosal mouse strains. Journal of Comparative Neurology, 1993, 336, 595-604.

**1992**

Lipp, H.P., and Wahlsten, D. Absence of the corpus callosum. In Driscoll, P. (Ed.), Genetically-Defined Animal Models of neurobehavioral Dysfunction. Birkhäuser-Boston, 1992, pp. 217-252.

Lassalle, J.M., and Wahlsten, D. Behavioral paradigms: General procedures and spatial memory. In: D. Goldowitz, D. Wahlsten and R.E. Wimer (Eds.), Techniques for the Generic Analysis of Brain and Behavior: Focus on the Mouse. Amsterdam: Elsevier, 1992, pp. 391-406.

Wahlsten, D. The problem of test reliability in genetic studies of brain-behavior correlation. In: D. Goldowitz, D. Wahlsten and R.E. Wimer (Eds.), Techniques for the Genetic Analysis of Brain and Behavior: Focus on the Mouse. Amsterdam: Elsevier, 1992, pp. 407-422.

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**1991**

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Wahlsten, D., Lassalle, J.M., and Bulman-Fleming, B. Hybrid vigour and maternal environment in mice. III. Hippocampal mossy fibres and behaviour. Behavioural Processes, 1991, 23, 47-57.

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**1990**

Wahlsten, D. Insensitivity of the analysis of variance to heredity-environment interaction. Behavioral and Brain Sciences, 1990, 13, 109-120. (Target article)

Wahlsten, D. Goals and methods: The study of development versus partitioning of variance. Behavioral and Brain Sciences, 1990, 13, 146-161. (Reply to commentators)

Cassells, B., Collins, R.L., and Wahlsten, D. Path analysis of sex difference, forebrain commissure area and brain size in relation to degree of laterality in selectively bred mice. Brain Research, 1990, 529, 50-56.

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**1989**

Wahlsten, D., and Smith, G. Inheritance of retarded forebrain commissure development in fetal mice: Results from classical crosses and recombinant inbred strains. Journal of Heredity, 1989, 80, 11-16.

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differentiation in BALB/c mice. Journal of Neurogenetics, 1989, 5, 61-76.

Wahlsten, D. Genetic and developmental defects of the mouse corpus callosum. Experientia, 1989, 45, 828-838.

Wahlsten, D. Science or prejudice? Behavioral and Brain Sciences, 1989, 12, 546-547. (Commentary on paper by J. P. Rushton)

Wainwright, P., Pelkman, C., and Wahlsten, D. The relationship between nutritional effects on preweaning growth and behavioral development in mice. Developmental Psychobiology, 1989, 22, 183-195.

**1988**

Lyons, J.P., and Wahlsten, D. Postnatal development of brain and behavior of shaker short-tail mice. Behavior Genetics, 1988, 18, 35-53.

Bulman-Fleming, B., and Wahlsten, D. Effects of a hybrid maternal environment on brain growth and corpus callosum defects of inbred BALB/c mice: A study using ovarian grafting. Experimental Neurology, 1988, 99, 636-646.

Wahlsten, D. Bias and sampling error in sex differences research. Behavioral and Brain Sciences, 1988, 11, 214. (Commentary on paper by C.P. Benbow.)

**1987**

Wahlsten, D., Blom, K., Stefanescu, R., Conover, K., and Cake, H. Lasting effects on mouse brain growth of 24 hr postpartum deprivation. International Journal of Developmental Neuroscience, 1987, 5, 71-75.

Wahlsten, D. Defects of the fetal forebrain in mice with hereditary agenesis of the corpus callosum. Journal of Comparative Neurology, 1987, 262, 227-241.

Wahlsten, D., and Bulman-Fleming, B. The magnitudes of litter size and sex effects on brain growth of BALB/c mice. Growth, 1987, 51, 240-248.

**1984**

Wahlsten, D. Each behavior is a product of heredity and experience. Behavioral and Brain Sciences, 1984, 7, 699-700. (Commentary on paper by B.F. Skinner.)

Wahlsten, D. Growth of the mouse corpus callosum. Developmental Brain Research, 1984, 15, 59-67.

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Psychology Abstracts, 1984, Vol. I, p. 14.

**1983**

Wahlsten, D. Maternal effects on mouse brain weight. Developmental Brain Research, 1983, 9, 215-221.

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**1982**

Wahlsten, D. Deficiency of corpus callosum varies with strain and supplier of the mice. Brain Research, 1982, 239, 329-347.

Wahlsten, D. Review of "Intelligence, Heredity and Environment" by Philip Vernon and "The IQ Game" by Howard Taylor. Canadian Journal of Psychology, 1982, 35, 361-362.

Wahlsten, D. Mode of inheritance of deficient corpus callosum in mice. Journal of Heredity, 1982, 73, 281-285.

Wahlsten, D. Genes with incomplete penetrance and the analysis of brain development. In I. Lieblich (Ed.) Genetics of the Brain. Amsterdam: Elsevier, 1982, pp.367-391.

Silver, J. Lorenz, S.E., Wahlsten, D., and Coughlin, J. Axonal guidance during development of the great cerebral commissures: Descriptive and experimental studies, in vivo, on the role of preformed glial pathways. Journal of Comparative Neurology, 1982, 210, 10-29.

Wahlsten, D. Mice in utero while their mother is lactating suffer higher frequency of deficient corpus callosum. Developmental Brain Research, 1982, 5, 354-357.

**1981**

Wahlsten, D. Indeterminacy is inherent in an inadequate model of evolution, not in nature. Behavioral and Brain Sciences, 1981, 4, 255-257. (Commentary on paper by Plotkin and Odling-Smee.)

Wahlsten, D. Pre-natal schedule of appearance of mouse brain commissures. Developmental Brain Research, 1981, 1, 461-473.

Wahlsten, D. Review of "The IQ Game" by Howard Taylor. Behaviorists for Social Action Journal, 1981, 3, 33-34.

**1980**

Wahlsten, D. Review of Fuller and Thompson's "Foundations of Behavior Genetics." Canadian Journal of Psychology, 1980, 33, 281-284.

Wahlsten, D. Race, the heritability of IQ, and the intellectual scale of nature. Behavioral and Brain Sciences, 1980, 3, 358-359. (Commentary on book by A. Jensen.)

Wahlsten, D. A systems approach to the understanding of heredity, brain and behavior. Behavior Genetics, 1980, 10, 500 (abstr.).

**1979**

Wahlsten, D. A critique of the concepts of heritability and heredity in behavioral genetics. In J.R. Royce and L. Mos (Eds.), Theoretical Advances in Behavioral Genetics. Alphen aan den Rijn, The Netherlands: Sijthoff and Noordhoff, 1979, 425-481.

Wahlsten, D. Some logical fallacies in the classical ethological point of view. Behavioral and Brain Sciences, 1979, 2, 48-49. (Commentary on paper by I. Eibl-Eibesfeldt.)

Wahlsten, D., and Anisman, H. A note on the Roman and Tryon selected lines of rats. Behavior Genetics, 1979, 9, 325-326. (Letter in reply to letter by P. Broadhurst.)

**1978**

Wahlsten, D. Behavioral genetics and animal learning. In H. Anisman and G. Bignami (Eds.), Psychopharmacology of Aversively Motivated Behaviors. New York: Plenum, 1978, 63-118.

**1977**

Wahlsten, D. Heredity and brain structure. In. A. Oliverio (Ed.), Genetics, Environment and Intelligence. Amsterdam: Elsevier, 1977, 93-115.

Wahlsten, D., and Wainwright, P. Application of a morphological time scale to hereditary differences in prenatal mouse development. Journal of Embryology and Experimental Morphology, 1977, 42, 79-92.

Jones, G.B., Wahlsten, D., and Blom, G. A precision stereotaxic procedure for the mouse (Mus musculus): Method and instrumentation. Physiology and Behavior, 1977, 19, 445-448.

**1976**

Wahlsten, D., and Anisman, H. Shock-induced activity changes, adrenal lipid depletion and brain weight in mice: A genetic study. Physiology and Behavior, 1976, 16, 401-406.

**1975**

Wahlsten, D. Genetic variation in the development of mouse brain and behavior: Evidence from the middle postnatal period. Developmental Psychobiology, 1975, 8, 371-380.

Anisman, H., Wahlsten, D., and Kokkinidis, L. Effects of d-amphetamine and scopolamine on activity before and after shock in three mouse strains. Pharmacology, Biochemistry and Behavior, 1975, 3, 819-824.

Wahlsten, D., Hudspeth, W.J., and Bernhardt, K. Implications of genetic variation in mouse brain structure for electrode placement by stereotaxic surgery. Journal of Comparative Neurology, 1975, 162, 519-532.

**1974**

Wahlsten, D. Heritable aspects of anomalous myelinated fibre tracts in the forebrain of the laboratory mouse. Brain Research, 1974, 68, 1-18.

Anisman, H., and Wahlsten, D. Response initiation and directionality as factors influencing avoidance performance. Journal of Comparative and Physiological Psychology, 1974, 87, 1119-1128.

Padeh, B., Wahlsten, D., and De Fries, J.C. Operant discrimination learning and operant bar-pressing rates in inbred and heterogeneous laboratory mice. Behavior Genetics, 1974, 4, 383-393.

Wahlsten, D. A developmental time scale for postnatal changes in brain and behavior of $B6D2F_2$ mice. Brain Research, 1974, 72, 251-264.

Griffiths, D., and Wahlsten, D. Interacting effects of handling and d-amphetamine on avoidance learning. Pharmacology, Biochemistry and Behavior, 1974, 2, 439-441.

**1973**

Wahlsten, D. Contributions of the genes albinism (c) and retinal degeneration (rd) to a strain-by-training procedures interaction in avoidance learning. Behavior Genetics, 1973, 3, 303-316.

**1972**

Wahlsten, D., and Cole, M. Classical and avoidance training of leg flexion in the dog. In A.H. Black and W.F. Prokasy (Eds.), Classical Conditioning II. New York: Appleton-Century- Crofts, 1972, 379-408.

Wahlsten, D. Phenotypic and genetic relations between initial response to electric shock and rate of avoidance learning in mice. Behavior Genetics, 1972, 2, 211-240.

Wahlsten, D. Genetic experiments with animal learning: A critical review. Behavioral Biology, 1972, 7, 143-182.

**1969**

Wahlsten, D., and Sharp, D. Improvement of shuttle avoidance by handling during the intertrial interval. Journal of Comparative and Physiological Psychology, 1969, 67, 252-259.

**1968**

Wahlsten, D., Cole, M., Sharp, D., and Fantino, E. Facilitation or bar-press avoidance by handling during the intertrial interval. Journal of Comparative and Physiological Psychology, 1968, 65, 170-175.

Cole, M., and Wahlsten, D. Response-contingent CS termination as a factor in avoidance conditioning. Psychonomic Science, 1968, 12, 15-16.

Freckleton, W.C., and Wahlsten, D. Carbon dioxide-induced amnesia in the cockroach, Periplaneta americana. Psychonomic Science, 1968, 12, 179-180.

**1967**

Wahlsten, D., Cole, M., and Fantino, E. Is a stimulus associated with the escape from shock a positive or negative reinforcer? Study I. Psychonomic Science, 1967, 8, 283-284.

Wahlsten, D., Cole, M., and Fantino, E. Is a stimulus associated with the escape from shock a positive or negative reinforcer? Study II. Psychonomic Science, 1967, 8, 285-285.

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***Invited Presentations***:

April, 1973. "Heritable aspects of anomalous myelinated fibre tracts," Florida State University, Psychology Department.

June, 1973. "Conceptual and methodological problems in the genetic analysis of motivation and learning," invited symposium presentation, Canadian Psychological Association, Victoria, B.C.

November, 1973. "Genetics and development," Institute for Behavioral Genetics, University of Colorado.

February, 1974. "Genetics and the dynamics of mouse development, "The Jackson Laboratory, Bar Harbor, Maine.

April, 1974. "Motivational contributions to genetic differences in learning ability," McGill University, Psychology Department.

January, 1975. "Genetics of mouse brain development," McMaster University, School of Medicine.

February, 1975. "Demystification of behavioural genetics." Carleton University, Psychology Department.

October, 1978. "A critique of the concepts of heredity and heritability in behavioral genetics," invited paper, NATO Advanced Study Institute on Theoretical Advances in Behavior Genetics, Banff, Alberta.

Fall, 1978. Psychology Department, Queen's University.

April, 1979. "Genetics and intelligence," Department of Psychology, University of Quebec at Trois Rivieres.

August, 1981. "Agenesis of corpus callosum," Department of Psychology, University of Quebec at Trois Rivieres.

March, 1982. "Heredity and brain development," Department of Psychology, University of Waterloo.

September, 1982. "Hereditary defects of brain development: Agenesis of corpus callosum," Zoology Department, University of Iowa.

February, 1983. "Racism and science," Institutional Racism Seminar, University of Illinois, Champaign-Urbana.

February, 1983. "Heredity, brain development and learning ability," Department of Psychology, University of Illinois, Champaign-Urbana.

March, 1983. "A critique of the heritability coefficient," Department of Botany, University of Toronto.

November, 1983. "Prenatal origins of the corpus callosum and its agenesis," Developmental and Cellular Neurobiology Seminar, McMaster University.

November, 1983. "Problems of heritability analysis in non-laboratory populations," Population Biology Seminar, Columbia University, New York.

November, 1983. "Heredity and brain development," Department of Biology, York University.

May, 1984. "Genetics and brain development," invited symposium address, University of Waterloo Conference on Child Development, Waterloo, Ontario.

May, 1984. "Can there be effective screening for a multifactorial disorder?," invited symposium address, Behavior Genetics Association, Bloomington, Indiana.

June, 1984. "Do genes determine brain structure?," Department of Chemistry, University of Guelph.

September, 1984. "Heredity and development of the nervous system," invited symposium address, XXIII International Congress of Psychology, Acapulco, Mexico.

February, 1985. "Critique of recent genetic theories of human behavioral development," Developmental seminar, Department of Psychology, Brock University.

November, 1985. Keynote speaker, symposium on "Racism and academic freedom," University of Western Ontario.

April, 1987. "Biological aspects of social behaviour," invited symposium address, Canadian Society for the History and Philosophy of Science, Guelph, Ontario.

November, 1987. "Motivation and associative learning ability: Are these genetically independent processes?" Invited symposium presentation, Psychonomic Society, Seattle.

December, 1987. "Agenesis of the corpus callosum: The congenital split brain mouse," Department of Psychology, University of Connecticut, Storrs.

August, 1988. "Screening for genetic variants affecting corpus callosum in mice." Invited workshop address, International Congress of Genetics, Toronto.

January, 1989. 1) "Current trends in behavior genetics." 2) "Insensitivity of the analysis of variance to heredity-environment interaction." 3) "Is there a genetic program for development?" Centre for Advanced Study in Theoretical Psychology, University of Alberta.

January, 1989. "Randomness in prenatal brain development," Division of Neurosciences, University of Alberta.

January, 1989. "The rate of organismic development: Measurement, control and significance," Department of Zoology, University of Alberta.

January, 1989. "Is there an innate kernel of inborn capacities?" Developmental Psychology group, University of Alberta.

March, 1989. "Limitations of genetic explanation in education," Department of Educational Foundations, University of Alberta.

March, 1989. "Emergent properties of interacting systems," Department of Sociology, University of Alberta.

June, 1990. "Behavioral consequences of absent corpus callosum in inbred mice," invited symposium presentation, Behavior Genetics Association, Aussios, France .

November, 1990. "Three sources of individual differences", Department of Psychological Sciences, Purdue University.

February, 1991. Invited presentation at symposium on "What is wrong with race research?" sponsored by African Students Association, University of Western Ontario.

April, 1991. "Molecular biology requires a reformulation of the nature-nurture question in developmental psychology," invited symposium presentation, Society for Research on Child Development, Seattle.

August, 1991. "Defects of the fetal forebrain in acallosal mice," invited presentation at the IBRO Satellite Symposium on Callosal Agenesis, Quebec City.

August, 1991. "Genetic aspects of callosal development," invited presentation at the Second Dartmouth International Conference on the Corpus Callosum and Epilepsy, Hanover, New Hampshire.

September, 1991. "Randomness in prenatal brain development", Medical Genetics Research Group, Alberta Children's Hospital, Calgary.

October, 1991. "Congenital split brain mice", Department of Psychology, University of Lethbridge.

November, 1991. "The Intelligence of Heritability", Invited keynote address, Joseph R. Royce Research Conference, Department of Psychology, University of Alberta.

July, 1992. "Developmental genetics of absent corpus callosum," invited presentation, International Congress of Psychology satellite meeting on Corpus Callosum and Interhemispheric Transfer, Turnhout, Belgium.

October, 1992. "Gene-associated random variability in brain development." Invited symposium address at the Jacques Monod Conference on Genetics, Neurogenetics and Behavior. Aussois, France.

February, 1993. "Genetic and developmental studies of absent corpus callosum". Division of Neuroscience Seminar, University of Alberta.

April, 1993. "The Intelligence of Heritability", Department of Psychology, University of North Carolina, Greensboro.

May, 1993. "Genetics of absent corpus callosum." Genetics rounds, Division of Medical Genetics, Department of Pediatrics, University of Alberta.

November, 1993. "Behavioral genetics as radical reductionism." Invited symposium address, International Society for Developmental Psychobiology, Alexandria, Virginia.

March, 1994. "Congenital split brain mice: genes and randomness." Invited seminar, Laboratoire d'Ethologie et Psychologie Animale, Université Paul Sabatier, Toulouse, France.

March, 1994. "Single gene effects and brain-behavior correlation." Invited symposium address, CNRS conference on Cognition and Evolution, Ile de Berder, France.

April, 1994. "Standardizing and validating tests of mouse behavior: genetic aspects." Invited address at Banbury Center workshop on Genetics of Learning and Memory, Cold Spring Harbor Laboratory, New York

July, 1994. "Random variability in brain structure and behaviour: A third source of individual differences." Invited address, Canadian Psychological Association, Penticton, B.C.

February, 1995. "Some questions about the quantitative trait locus method." Invited symposium presentation, AAAS annual meeting, Atlanta, Georgia.

February, 1995. "Heredity-environment interaction: a critique." Invited colloquium and workshop, Center for Developmental Science, University of North Carolina, Chapel Hill.

February, 1995. "The nature-nurture question: some general conclusions.: Invited colloquium, Department of Psychology, University of Illinois.

February, 1995. "Congenital split brain mice." Invited colloquium, Beckman Institute, University of Illinois.

June, 1995. "Artifical selection of recombinant inbred strains: a powerful tool for examining brain-behavior relations." Invited symposium presentation at Behavior Genetics Association satellite meeting on Heredity, Nervous System and Behavior. Richmond, Virginia.

June, 1995. Invited participant, workshop on Animal Models of Psychiatric Diseases: to Man from Mouse. MacArthur Foundation research network on psychopathology and development, Chicago.

September, 1995. "False alarm raised by the genetic clapper in *The Bell Curve*." Dept. of Psychology monthly Psychoquium, University of Alberta.

October, 1995. "Principles of behavioral and neural genetics." Invited presentation at the Jacques Monod Conference on Genetics, Neurogenetics and Behavior II, La Londe-les-Maures, France.

October, 1995. Lectures on "Neurogenetics" and "Testing animal behavior" plus lab on "CNS histology and morphometry." First French-American Summer School on Neurobehavioral Genetics, University of Paris and CNRS, Paris, France.

March, 1996. "Developmental genetic studies of congenital split brain mice." Dept. of Human Anatomy and Medical Neurobiology, Division of Neuroscience, Texas A&M University.

June, 1996. Lectures on "Neurogenetics" and "Statistical power analysis" at the Second Franco-American Summer School on Neurogenetics, Pennsylvania State University.

March, 1997. "Sex differences in the human corpus callosum: myth or reality?" Grant MacEwan College, Edmonton.

September, 1997. Lectures on "Statistics and experimental design" and "Genetic approach to functional morphology," Third French-American Summer School on Neurobehavioral Genetics, CNRS, Orléans, France.

June, 1998. Lectures on "Statistics and experimental design," Fourth French-American Summer School on Neurobehavioral Genetics, Greeley, Colorado.

November, 1998. "Multisite trials and validation issues," Brain Research Interactive conference, San Diego, California.

May, 1999. Mouse Strain Characteristics Database Summit, Bar Harbor, Maine.

June, 1999. "Large scale screening for mutations altering mouse behaviour - pitfalls and prospects." Symposium on Behaviour Genetics, Canadian Society for Brain, Behaviour, and Cognitive Science, Edmonton.

September, 1999. "Absence of the corpus callosum: Genes, environment and a third source of developmental errors." Oregon Health Sciences University, Portland.

October, 1999. "Mouse behavior genetics: interactions with the laboratory environment." National Institute of Mental Health, Rockville, MD.

October, 1999. "Absent corpus callosum: axon guidance and developmental thresholds." Department of Anatomy, University of Wisconsin, Madison.

November, 1999. "Behavior genetics into the 21st century." Department of Psychology, Florida State University, Tallahassee.

February, 2000. "Standardizing tests of mouse behaviour: reasons, recommendations and reality." Opening keynote address for 3-day symposium on Behavioural Phenotyping of Mouse Mutants, University of Cologne, Germany.

May, 2000. "Behavior genetics." Third International Institute on Developmental Science, Chapel Hill, NC.

August, 2000. "Behavioral genetics into the 21st century." American Psychological Association, invited address to Developmental division, Washington DC.

August, 2000. "Learning lab" and "Gene x environment interaction." Sixth International Behavioral Neurogenetics Summer School, Portland, OR.

November, 2000. "Randomness in development: a third source of individual differences and novelty." Wiley lecture, International Society for Developmental Psychobiology, New Orleans.

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***Voluntary conference presentations*:**

May, 1967. "Is a stimulus associated with escape from shock a positive or negative reinforcer?" Western Psychological Association, San Francisco (with Michael Cole).

September, 1967. "Description of freezing and avoidance conditioning", "Classical and instrumental components of defense conditioning," Psychonomic Society, Chicago (with Michael Cole).

May, 1969. "Classical and avoidance training of leg flexion in dogs", invited presentation, Classical conditioning conference, McMaster University (with Michael Cole).

April, 1973. "Heritable aspects of anomalous myelinated fibre tracts," Behavior Genetics Association, Chapel Hill, North Carolina.

November, 1973. "Genetic variation in status of postnatal development," Society for Neuroscience, San Diego, California.

June, 1974. "Genetics and prenatal development of mouse brain," Behavior Genetics Association, Minneapolis, Minnesota.

January, 1975. "Genetic bases of racial differences in IQ," McMaster University conference on developmental psychology.

June, 1978. "Hereditary deficiency of corpus callosum in mice: Lack of segregation within a BALB substrain showing incomplete penetrance," Behavior Genetics Association, Davis, California.

June, 1979. "Mode of inheritance of deficient corpus callosum in the brains of laboratory mice," Behavior Genetics Association, Middletown, Connecticut.

June, 1980. "A systems approach to the understanding of heredity, brain and behavior," Behavior Genetics Association, Chicago, Illinois.

October, 1981. "Mice in utero while their mother is lactating suffer higher frequency of defective corpus callosum," Society for Neuroscience, Los Angeles, California.

April, 1982. "The heritability of behavior viewed in the light of ecological principles," Ontario Ecology and Ethology Colloquium, University of Guelph.

June, 1982. Symposium on Ethical Issues. "Social equity-philosophical considerations," Behavior Genetics Association, Ft. Collins, Colorado.

November, 1982. "Spontaneous occurrence of a mutation causing severe defects of cerebellum and motor coordination," Society for Neuroscience, Minneapolis, Minnesota (with J.P. Lyons and W. Zagaja).

June, 1983. "Pre- and postnatal growth of the mouse corpus callosum," Southern Ontario Neuroscience Association, McMaster University, Hamilton.

July, 1983. "Mouse strains differ in responses to food deprivation," Behavior Genetics Association, University of London, England.

November, 1983. "Structural changes in the brains of mice with agenesis of corpus callosum," Society for Neuroscience, Boston, Massachusetts (with G.B. Jones).

July, 1985. "Anatomy and development of the hippocampus and dentate gyrus in the shaker short-tail (sst) mutant mouse," American and Canadian Association of Anatomists, Toronto (with R.S. Nowakowski).

October, 1985. "Defects of the glial sling in mouse fetuses with hereditary absence of corpus callosum," Society for Neuroscience, Dallas, Texas.

October, 1985. "Asymmetric development of the hippocampal region in the shaker short-tail (sst) mutant mouse," Society for Neuroscience, Dallas, Texas (with R.S. Nowakowski).

June, 1986. "Heritable aspects of coordination of axon outgrowth: A genetic analysis of collision of anterior commissure and columns of the fornix in mouse brain," Southern Ontario Neuroscience Association, Guelph, Ontario (with B. Cassells).

November, 1986. "Corpus callosum defects and maternal environment in the BALB/c mouse: Effects of ovarian grafting," Society for Neuroscience, Washington, D.C. (with B. Bulman-Fleming).

May, 1987. "Effect of heredity and early environment on brain weight and myelinated forebrain fibre tracts of laboratory mice," Southern Ontario Neuroscience Association, Toronto (with P.W. McDonald).

June, 1987. "Three sources of individual differences," theory review, Canadian Psychological Association, Vancouver.

June, 1987. "Mechanisms of axonal guidance in mouse forebrain: Redundancy and competition," Canadian Psychological Association, Vancouver (with B. Cassells).

June, 1987. "Insensitivity of analysis of variance to heredity-environment interaction," Behavior Genetics Association, Minneapolis, Minnesota.

June, 1987. "Variation in spatial knowledge in Mus musculus: Maternal and genetic effects," Behavior Genetics Association, Minneapolis, Minnesota (with J.M. Lassalle and B. Bulman-Fleming).

November, 1987. "Retarded fetal growth of forebrain commissures in BALB/c mice: Mode of inheritance," Society for Neuroscience, New Orleans (with G. Smith).

November, 1987. "Dendritic and axonal abborization of ectopic pyramidal cells in the hippocampus of the dreher mutant mouse," Society for Neuroscience, New Orleans (with M. Sekiguchi and R.S. Nowakowski).

November, 1987. "Brain weight and corpus callosum size in mice selected for high and low degrees of paw preference," Society for Neuroscience, New Orleans (with B. Cassells and R.L. Collins).

November, 1987. "Hybrid vigor and maternal environment effects on mouse brain size," Society for Neuroscience, New Orleans (with B. Bulman- Fleming and J.M. Lassalle).

November, 1987. "Sex differences in the corpus callosum," Southern Ontario Neuropsychology Group, University of Waterloo.

November, 1988. "Hippocampal mossy fibre distribution in relation to tests of spatial memory in mice," Society for Neuroscience, Toronto (with J.M. Lassalle and B. Bulman-Fleming).

November, 1988. "Severity of corpus callosum deficits in two substrains of BALB/c Wah mice in relation to uterine location of fetuses," Society for Neuroscience, Toronto (with B. Bulman-Fleming).

October, 1989. "Patterns of cerebellar foliation in recombinant inbred mice," Society for Neuroscience, Phoenix, Arizona (with M. Andison).

October, 1989. "Heredity of collisions between anterior commissure and columns of the fornix in mouse brain," Society for Neuroscience, Phoenix, Arizona (with B. Cassells).

May, 1990. "Power and n required to detect interaction in a 2 x 2 design," Canadian Psychological Association, Ottawa.

November, 1990. "Commissure formation in six mouse strains with absent corpus callosum," Society for Neuroscience, St. Louis (with B. Bulman-Fleming).

November, 1990. "Tests of genetic allelism between four inbred mouse strains with absent corpus callosum". Society for Neuroscience, St. Louis (with D. Livy).

June, 1991. "The first traverse of callosal axons observed with the carbocyanine dyes DiI and DiA," Canadian Society for Brain, Behavior and Cognitive Science, Calgary (with H.S. Ozaki).

October, 1992. "Prenatal development of callosal axons in acallosal strain mice: A quantitative study with carbocyanine dyes." Society for Neuroscience, Anaheim (with H.S. Ozaki).

June, 1994. "Sex differences in the human corpus callosum: myth or reality?" Brain, Behaviour and Cognitive Science meeting, Vancouver (with K. Bishop).

September, 1994. " A three cut surgical approach for callosotomy in the adult mouse." European Neuroscience Association, Vienna, Austria (with P. M. Schalomon).

November, 1994. "The postnatal development of the aberrant longitudinal bundle and the corpus callosum in BALB/cWahl mice." Society for Neuroscience, Miami (with S. L. Schmidt and N. Parra).

November, 1995. "An increase in anterior commissure axon numbers in acallosal mice." Society for Neuroscience, San Diego. (with D. J. Livy and M. Schalomon).

November, 1995. "New recombinant inbred strains expressing 100% total absence of the corpus callosum." Society for Neuroscience, San Diego. (with V. Sparks).

October, 1997. "Critical events at the telencephalic midline in acallosal mouse embryos." Society for Neuroscience, New Orleans. (with K. M. Bishop)

October, 1999. Proof of a third source of individual differences in brain structure that is neither hereditary nor environmental. International Behavioural and Neural Genetics Society, Kay Largo.

October, 1999. Behavioral effects of absent corpus callosum and the 5HT1B knockout in strain 129 mice. Society for Neuroscience, Miami. (With P. M Schalomon, J. C. Crabbe, B. C. Dudek)

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***Theses Supervised***

**University of Waterloo:**

1970 Jonathan B. Kronick (M.A.) "Teratogenic effects of ethyl alcohol in the mouse"

1971 Carolyn L. Brain (Ph.D.) "Genotypic odor-type and agonistic behavior in the mouse"

1971 Marilyn Brecher (M.A.) "Internal-external locus of control, verbal fluency and creativity in children"

1972 David L. Weening (Ph.D.) "Pentylenetetrazol-induced convulsions: delayed development of retrograde amnesia"

1972 Hymie Anisman (Ph.D.) "Effects of signaled inescapable shock on the retention of aversively motivated behavior: role of response repertoire changes" (co-supervisor)

1973 Kenneth Roy Keeling (M.A.) "Hypnosis and adaptive regression: a study using Wild's cognitive shift measure"

1974 Patricia Wainwright (M.A.) "The effects of heterosis on the rate of prenatal development in the laboratory mouse"

1975 G. Brian Jones (M.A.) "Spatial organization of mouse forebrain: six strains compared by a new stereotaxic technique"

1977 Patricia E. Wainwright (Ph.D.) "Differences in the maternal performance of inbred and hybrid laboratory mice (Mus musculus)"

1979 George Brian Jones (Ph.D.) "Plasticity of neural projections from cerebral cortex in mice with hereditary agenesis of corpus callosum: a lesion-degeneration study"

1980 David DiBattista (Ph.D.) "Role of the hypothalamus in the sympathoadrenal response to 2-deoxyglucose"

1983 Jill P. Lyons (M.A.) "Postnatal brain and behavioral development of hybrid mice homozygous for the shaker short-tail (sst) gene"

1986 Heather Anne Cake (M.A.) "Heterosis and effects of postpartum deprivation on brain and body growth"

1988 Samuel Bryan Cassells (Ph.D.) "Hereditary influences on the morphology of anterior commissure and columns of fornix in Mus musculus"

1988 Mary Barbara Bulman-Fleming (Ph.D.) "Maternal environment and deficiency of corpus callosum in BALB/c mice"

1990 Daniel Joseph Livy (M.A.) "Test of genetic allelism between four inbred mouse strains with absent corpus callosum"

**University of Alberta:**

1996 Daniel Joseph Livy (Ph.D.) "Interhemispheric axon pathway development in mice with agenesis of the corpus callosum" (co-supervisor with S. K. Malhotra)

1997 Christopher Dean (M.Sc.) "Symmetry of mouse corpus callosum development"

1997 Katherine Mary Bishop (Ph.D.) "A threshold model for development of the corpus callosum in normal and acallosal mice"

1999 Petra Melike Schalomon (Ph.D.) Motor coordination in congenitally acallosal and callosotomized mice

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***Referee for Journal Articles*** (various years):

American Psychologist
Anatomy and Embryology
Animal Learning and Behavior
Behavioral Brain Research
Behavior Genetics (member of Editorial Advisory Board)
Behavioral and Brain Sciences
Behavioral and Neural Biology
Behavioural Processes
Brain Research
Brain Research Bulletin
Canadian Journal of Psychology
Developmental Psychobiology
European Bulletin of Cognitive Psychology
European Journal of Neuroscience
Experimental Neurology
Genetica
Italian Journal of Psychology
Journal of Comparative Neurology
Journal of Comparative Psychology
Journal of Heredity
Journal of the Institute for Laboratory Animal Research
Journal of Neurogenetics (member of editorial advisory board)
Journal of Personality and Social Psychology
Laterality
Learning and Motivation
Mental Retardation and Developmental Disabilities Research Review
Neurobiology of Learning and Memory
Pharmacology, Biochemistry and Behavior
Physiology and Behavior
Proceedings of the National Academy of Science U.S.A.
Psychobiology
Psychological Bulletin

Science

***Reviews of Grant Proposals*** (various years):

Alberta Mental Health Research Committee (member)
Alberta Children's Hospital Foundation
Canadian Genome Analysis and Technology program
Canadian Heart Foundation
Manitoba Health Sciences Centre Foundation
MRC operatng grants; internal review member; site visit for development grant
NSERC operating and collaborative grants
NIDA (U.S.A.) - initial review group for RFA
NIMH (U.S.A.) Operating grants and Initial Review Group
NSF (U.S.A.)
SSHRCC strategic grant

***Departmental Service*** (various years at University of Waterloo):

Animal care advisory committee
Biopsychology division chairman
Chairman selection committee
Colloquium committee
Planning committee
Salary, promotion and tenure committee
Shop review committee
Statistics review committee

***University Service*** (various years at University of Waterloo):

Committee on Animal Care (vice-chairman, 1978)
Planning committee, Department of Man-Environment

***Departmental Service*** (various years at University of Alberta):

Faculty of Science fund raising representative
Undergraduate curriculum committee
Animal Ethics Committee, Chair
Chair's Advisory Committee, elected member 1992-94
Chair Selection Committee, elected member 1996-98

***University Service*** (various years at University of Alberta):

AAS:UA Council
AAS:UA External Relations Committee
Centre for Gerontology Board of Directors
Ad hoc committee to review Biosciences Animal Service (1993)
Faculty of Science Academic Appeals Committee
Division of Neuroscience committee to establish a Neuroscience Hounours program
Division of Neuroscience planning committee, 1999-

# Critique of Perchlorate Study

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23 Apr '01

# PART 1: CRITIQUE OF THE NEUROMORPHOLOGY STUDIES

## Summary

In the original study (Argus 1998) and in the present study (Primedica 2001) the offspring of female rats exposed to differing doses of perchlorate in their drinking water during gestation were evaluated on a number of biological parameters. The corpus callosum (CC), the major fiber tract connecting the left and right brain hemispheres, was one of the key neuromorphological targets studied. In both studies animals exposed to perchlorate during pregnancy had CC values significantly different from controls. However, there were insufficient samples of the CC in both studies, and flawed measurement in the second study. Thus, it is not possible to make any meaningful interpretation of the empirical findings.

## Introduction

In this review I will focus primarily upon the corpus callosum, since that is the only brain structure that has been examined in both the Argus 1998 and the Primedica 2001 studies, and is a structure with which I have expert knowledge and experience. In this section I present some background material on the corpus callosum, followed by several broad conclusions.

The corpus callosum (CC) is not a "structure" in the same sense as the kidneys, lungs, brain, gonads, etc. are structures. The CC does not actively participate in physiological functions. Instead, it is merely a "bridge" which allows neural processes originating in one brain hemisphere to cross over to the opposite hemisphere (Wahlsten, 1982). The axons crossing the CC bridge always originate in cortical areas. None originate in the CC.

In most instances homotopic connections are made in the opposite hemisphere, but occasionally the axons go to heterotopic regions. The fibers going through the CC roughly follow an anterior-posterior gradient. Brain regions in the anterior portion of the brain (e.g., prefrontal cortex) send their processes through the genu of the CC, while fibers from the occipital cortex course through the splenium; and other cortical regions occupy intermediate positions along the CC. However, for the rat no one has yet determined exactly where fibers from different cortical areas enter the CC.

Because of these characteristics, there are major advantages and disadvantages in studying the CC. The disadvantage is that the CC is extremely heterogeneous, and measurements taken from one portion cannot be generalized to other parts of the CC. Thus, to "sample" the CC requires multiple measurements. The advantage is that the CC may be thought of as a two-dimensional portrayal of the three-dimensional brain, analogous to the relationship between a map and the terrain it depicts. As an example, a number of studies have found that male and female rats differ significantly in their CC measurements in different regions with the major differences found in the genu and splenium (Denenberg et al., 1991; Fitch et al., 1990; Fitch and Denenberg, 1998), thus suggesting that there are sex differences in the neuroanatomy of the prefrontal and occipital cortices. The key point here is that a significant difference in a particular CC region implies that the difference really exists in the cortical area sending fibers through that CC region.

Several methods have been proposed for obtaining CC measurements. All these procedures start with a sagittal section of the callosum (though coronal sections can also be used) and assume the CC can be treated as a linear structure. The usual measurement of the CC is a "width" score obtained along the dorsal-ventral axis (e.g., Clarke et al., 1989; Denenberg et al., 1991). Denenberg et al. specify 99 widths along the CC length [i.e., along the anterior-posterior (or genu-splenium) axis], whereas Clarke at al. have 27 widths. The CC is usually broken into a number of regions and the average width per region (or area of the region) is often used as a final score for statistical analysis. No one uses a single width score as a meaningful measure.

Finally, a number of factors contribute to the CC width or region measurements. The major ones are: number of axons crossing, the ratio of myelinated to unmyelinated axons, the packing density of the axons in the CC, differing amounts of non-neural material (e.g., glia) in the CC. EM studies are required to determine the reasons for differences found at the LM level (Juraska, 1988; Mack, 1995). EM studies of the CC were also recommended by the 1999 Peer Review Panel.

The summary above provides the context for two broad conclusions concerning the perchlorate studies.

• There is insufficient sampling of the CC to draw any meaningful conclusions about the effects upon the offspring of exposing pregnant rats to perchlorate in the drinking water.

• The CC measurement procedure employed is seriously flawed.

I discuss these issues in the following sections.

## Corpus Callosum Measurement and Analysis

The CC is one of the few structures which, we can confidently assert, is symmetrical. This is because, as noted above, the CC is simply a bridge and whatever goes in on one side comes out the other. Thus, there is no "right" or "left" corpus callosum. This is the flaw in the measurements made in the Primedica 2001 study. The proper measurement of the CC is at the midline of the coronal section,v using a minimum of 10 sections to obtain a series of measures.

Even though the measurements are flawed in this study, it might be possible to obtain some information, since the measures obtained would be expected to be proportional to the correct data. For the proportionality argument to hold up, it is necessary that the "right" and "left" measures (1) be highly correlated with each other, and (2) have the same mean scores.

Therefore, I entered the raw scores for the Day 10 and Day 22 right and left CC measures into a spreadsheet, along with the brain weight data. [These scores were taken from sections A2 and A3. To determine correlations, I used the "Pearson" function in Excel. For analysis of variance I used Statistica 4.1 for the Macintosh.]

The ANOVAs had Group (5 levels representing the doses administered) for the independent classification and CC at 2 levels (Right and Left) as a repeated measurement. Separate ANOVAs were run for males and females, as was done by the statistician who did these analyses; and then I put males and females together in a separate analysis.

Lactation Day 10 Analysis

The ANOVAs found that the left and right measurements did not differ from each other at any dose level for either sex (see Figs. 1 and 2).

The Pearson correlation of the Right and Left scores for the males was r = .887; for the females, r = .845.

These findings indicate that the measurements had high reliability (correlations) and reproducibility (means). Therefore, the Right and Left scores were averaged to yield one CC score.

An omnibus test of male Groups, using the averaged CC score, found significance. A Least Significant Difference (LSD) analysis determined that the Control group differed significantly from the 0.1 and 1.0 mg groups (see Fig. 3). A similar analysis of the female Group levels did not find significance via the omnibus test (Fig. 4).

The experimental design used male-female littermates. Therefore, an ANOVA was run with the classifications of Group (5 levels) and Sex (2 levels, repeated measures), and with the averaged CC score as the dependent variable. There was a significant Group effect, a significant Sex effect, and the Group x Sex interaction was significant (Fig. 5).

*Comment.* The Primedica 2001 study reported that the CC of pups whose mothers were treated with perchlorate at doses of 0.1 and 1.0 mg were statistically significant for the CC width measure of males, but did not affect the CC of females at any dose level. However, as presented above and later in this review, there are fundamental issues with the analytical procedures. Specifically. the measurement procedure is too meager to allow one to offer an interpretation of this empirical finding, though the data may be of use as preliminary findings. It is worth noting that the effect size (d = mean difference/SD) of the difference between the control group and the 0.1 and 1.0 perchlorate groups is approximately 1.4 SD units (see Fig. 3). This is a very large effect, since Cohen (1969) specified .8 SD units as a "large" effect.

Lactation Day 22 Analysis

The ANOVA of the male Right and Left scores found a significant Group x Right/Left interaction (Fig. 6). Inspection of the Figure indicates that differences were present for the Control group and for dose groups 1.0 and 30.0 mg (these differences were not tested for significance).

A similar analysis of the 22 Day females did not find any effect, though the Group x Right/Left interaction had $p < .10$ (Fig. 7).

The Pearson correlation of the Right and Left scores for the males was $r = .909$; for the females, $r = .901$.

The CC data of the females meet the requirements of reliability and reproducibility and the Right/Left scores were averaged to yield one CC value. Unfortunately, the significant interaction in the male data raise serious concerns since this signifies that there is asymmetry in the measurements on either side of the midline for the Control group and for the 1.0 and 30.0 perchlorate groups (see Fig. 6). Since the Right/Left reliability was high, and since other options were not available, the Right/Left scores were also averaged to yield one CC value.

An omnibus test of male Groups, using the averaged CC score, did not find significance (Fig. 8). A similar analysis of the female Groups found significance via the omnibus test (Fig. 9). An LSD analysis determined that the Control group differed significantly from the 0.01 and 30.0 mg groups. Note that these significant differences are in opposite directions.

The experimental design used male-female littermates. Therefore, an ANOVA was run with the classifications of Group (5 levels) and Sex (at 2 levels, repeated measures), and with the averaged CC score as the dependent variable. There was a significant Group effect and the Group x Sex interaction was significant (Fig. 10).

*Comment.* In contrast to the Day 10 data, where male controls differed from males getting perchlorate, at Day 22 the only differences found were with females. As with the Day 10 data, no interpretation of these findings can be offered. In addition, a disturbing finding is that the

CC scores of the Day 10 group is larger than the Day 22 scores (Figs. 5 and 10).

**Overall Evaluation and Recommendations of CC Studies**

The procedures and findings raise a number of questions, as follows:

• How was a decision originally reached to obtain only one measurement of the CC?

• In the original study (Argus 1998), the group doing the neuromorphometrics, Consultants in Veterinary Pathology (CVP), measured the corpus callosum at the midline. Why was that changed to "right" and "left" measurements in the second study (Primedica 2001)?

• Why didn't someone at CVP raise a question about a single measure of the CC?

• How did the 1999 Peer Review Panel that reviewed the Argus 1998 study fail to note that getting only a single CC measurement was almost useless?

• Why is there such a discrepancy between the Day 10 CC measurements obtained by CPV and the Day 22 CC measurements obtained by Experimental Pathology Laboratories (EPL)? Or did EPL obtain the brain weights? On p. 19 of the main text (last para) is the following: "Brains from pups sacrificed on DL 10 and DL 22 were shipped to Consultants in Veterinary Pathology and Experimental Pathology Laboratories, respectively...Brain weights of pups sacrificed on DL 22 were recorded postfixation by Consultants in Veterinary Pathology, Inc." I find this confusing since, presumably, EPL did the CC measurements.

• CPV also did the 12 day CC measurements in the Argus 1998 study. Below I give the averages for the control groups, summed over "right" and "left" and males and females:

Day 10: 326 (CPV)
Day 12: 295 (CPV)
Day 22: 224 (EPL)

The Days 10 and 12 numbers go together, but the Day 22 values are markedly off. Is this due to the measurements being obtained off the midline? Is this a sampling problem, a laboratory problem, or some other kind of problem? A discrepancy of this magnitude raises many questions, the most critical of which is: How do the other measurements from the laboratory that determined the brain weights and CC values of the Day 22 pups (EPL or CVP) compare to measurements obtained on similar groups of animals evaluated in other laboratories?

## Some Statistical Issues

In this section I wish to raise some questions concerning the statistical analyses.

Why is an omnibus F-test run prior to doing the Dunnett t-tests? The Dunnett procedure does not require a significant omnibus test, so I do not understand the rationale for doing this.

Why were there no trend or regression analyses to test for a dose-response curve? TERA did run some regression analyses, but they were based upon the group means and did not take individual variation into account. Examination of the data in tho tables on pages 56, 58, 66, and 67 suggest that a quadratic function might fit the scores. This is particularly true for the day 22 females, where 13 of the 22 measures can be viewed as U-shaped or as an inverted-U. There are also a number of possible quadratic fits in the other tables as well.

Since male-female littermate pairs were used in this study, that should be built into the statistical analysis. Normally I would recommend a Sex (2 levels) x Age (2 levels) x Dose (5 levels) ANOVA with repeated measures on the Sex variable. However, Age cannot be included because different labs did the measurements resulting in bizarre data with the Day 10 group having larger CC values than the Day 22 group. Therefore, one is limited to running a two-factor Sex x Dose design, with repeated measures on Sex. This would be done for each Age group separately. I do not recommend doing an omnibus test on the Dose dimension. Instead, I would test for Linear, Quadratic, and Cubic trends, and would extract the Sex x Linear, Sex x Quadratic, and Sex x Cubic interactions. These are far more powerful than doing separate analyses within sex using an omnibus test. In addition, in those analyses where Sex was not significant as a main effect or in interaction, I would recommend running the Dunnett test pooled over the two sexes.

On p. 23 of the text is the statement that Day 10 male rats had significantly higher scores on 15 linear dimensions. I only find higher scores on 9 dimensions, using the data in the table on p. 56. On the same page is the statement that Day 10 female rats had decreases in 18 of 19 measures. Examination of the data in the table on p. 58 find 11 significant differences, representing 8 measures, with 10 of the 11 having lower scores.

I noticed that Female Day 10 subject #16717 has CC scores 2 SDs from the mean. Are the data routinely inspected to look for outliers, or is there a program that does that?

In entering the raw scores for the analyses, I noticed that the vast majority of the Day 22 brain weight scores have a zero in the third decimal place. The next most frequent number is 5. There are only a few numbers other than 0 or 5. This is in contrast to the Day 10 data where the third decimal place has all possible numbers.

Several research groups have found that brain weight does not correlate with CC measures. Since I had these data available, I ran correlations of brain weight and average CC score within age groups and sex. None of the four correlations were significant. Thus, there is no need to consider adjusting the CC measures for brain weight.

To get a sense of the between-litter variance of the rats in the study, I correlated, within each age group, the brain weights of male and female littermates. The correlations were r = .697 for Day 10 rats and r = .298 for Day 22 rats (both significant). This indicates that there is still considerable variance in this population. The lower correlation for the Day 22 group may reflect greater diversity as the animals get older, or it may reflect the less precise brain weights (third decimal lacks significance).

The same littermate correlations were then done using their CC scores. Neither correlation was significant (Day 10, r = .04; Day 22, r = -.04). It's difficult to know what to make of this finding.

## Experimental Design Considerations

If this study is repeated (I think it should be), the following points should be seriously considered.

- The CC does not become fully mature until around 40 days of age. Thus, it is necessary to have one group of animals tested around 60-70 days or later to be certain that measures are obtained after the brain has matured.

- The Morris maze should be used as the test of spatial learning. There should be a minimum of 4 trials a day for 5 successive days. The variables measured should include time, distance, time in each annulus, and time in each quadrant. Day 6 should consist of a probe trial with the platform removed.

- Other cognitive measures should be included, with at least one measure of non-spatial associative learning (e.g., discrimination learning) and fear-based learning (e.g., two-way shuttlebox).

- A measure of affective behavior (open-field or plus-maze) needs to be included. If the open-field test is used, the animals need to be tested for 3 successive days, at a minimum.

- The initial stimulus for the perchlorate research were the findings concerning the thyroid. How would an expert in thyroid physiology judge the thyroid data at the dose levels used in these studies? It would be useful to include a dose level of perchlorate sufficiently high that everyone agrees this is toxic to the thyroid. Then one would be able to see how that dose affects brain development and behavior. In addition, and as a standard procedure, it would be wise to include a positive control (e.g., pregnant rats on an iodine deficient diet, or given different antithyroid agents, etc.) as an independent way of confirming any toxic effects seen with any of the doses of perchlorate.

• Someone who is an expert on CC histology and measurement should be brought in as a consultant to review the complete CC protocol from perfusion onward.

• Continue taking one male and one female from each litter. Ns of 13-16 are quite good when effects are large. Some thought should be given to a statistical power analysis. This would compel the design group to think about a minimal effect size, alpha level, and N per group.

## PART 2: ANSWERS TO QUESTIONS PRESENTED BY TERA

TERA listed a series of questions they wished considered. Though many of these have been answered in Part 1 above, I address these questions here.

**The first set of questions (in italics) and my answers follow.**

*Do the statistically significant changes observed in the Primedica (2001) study, when considered in light of the results of the Argus (1998) neurobehavioral developmental study, represent a consistent effects of perchlorate exposure on behavioral or structural development of the brain?*

*If these studies do demonstrate a consistent effect, is the effect biologically relevant?*

*If the observed effects are biologically relevant, can they be considered adverse, as defined in the discussion on page 3 of this cover letter?*

*If the effect on brain development on behavior is considered adverse, what dose produces the adverse effect in the test species (rat)?*

*If the effects are considered to be adverse, are they relevant to humans?*

The only behavioral data are from the Argus 1998 study. No effects were found for passive learning, an "M-test" for water maze learning, or acoustic startle. Greater motor activity was obtained in an open-field type of test. However, this effect apparently has not been replicated in the Primedica 2001 study, though additional statistical analyses are yet to be done.

Therefore, at the behavioral level, nothing substantial has been obtained. However, the sampling of possible behaviors represented by these tests is very narrow. For example, no measures were obtained of spatial learning (I am skeptical about the M-maze), non-spatial associative learning (e.g., discrimination learning), fear-based active learning (one-way or two-way shuttlebox), Pavlovian learning, novelty/exploratory behavior, or spatial and non-spatial working memory, to name a few important behavioral processes.

With respect to neuromorphology, the corpus callosum data are largely useless except to suggest that this fiber bundle needs to be examined carefully.

The question of consistency of brain effects is sex-specific. In males, at both 10 and 22 days, perchlorate acts to increase brain measures; whereas in females at the same two ages, those exposed to the chemical *in utero* have smaller brain measures. Since these data are highly suspect, I do not think much can be said about consistency until the study is re-done (and re-done correctly).

Are these biologically relevant? First, the experiment and analyses must be designed and conducted correctly. Then I think one has to assume relevance for any change in a brain region that is known to play a key role in behavior. Further, the findings suggest that the mechanisms of action may differ in the two sexes.

**The next set of questions revolved around the design of the Primedica 2001 study. Below I list the relevant questions and my comments.**

*Please comment on aspects of the experimental design that would affect the interpretation of the brain morphometry results.*

The sampling from the CC is inadequate, as noted in Part 1.

*Were the morphometric methods used appropriate for determining an effect on brain development?*

Not for the corpus callosum.

Further, measures at two time points (Days 10 and 22) is not sufficient to determine a developmental path.

*Do the measurements made in this study have inherent sources of variation, and was the inherent variability adequately controlled in the study? How does the variability reported in this study compare with the expected variance?*

There is always sampling error ("inherent variability") in any study. The descriptions of the protocols followed in breeding females, selecting which females to use, selecting pups, etc. all appear quite sound. The Ns of 13-16 per group is certainly within acceptable limits.

The use of one male and one female per litter is excellent. However, the statistical analyses would have been more efficient if male/female littermate pairs had been put into the same ANOVA.

*Did the techniques used result in adequate homology in the brain sections and did they adequately control for sources of variation in the brain morphometric measurements?*

Drs. Juraska and Wahlsten can provide you with a better answer than I can.

*How does the use of coronal sections affect the ability to compare the results with values reported in the literature?*

Nearly all of the studies published in peer-reviewed literature measure the CC from sagittal sections. However, if done correctly coronal sections should have no effect upon the CC measures. Indeed, I have a colleague who argues that coronal sections are better than sagittal ones because the plane of the cut is very critical in a sagittal section and much less so in a coronal section. To do this correctly, it is necessary to obtain multiple coronal slices along the anterior-posterior axis to ensure adequate sampling throughout the CC (in effect, this is just like measuring the CC from a sagittal plane since you one is still measuring coronal sections along the entire length of the CC).

*How do the linear dimensions of measured brain regions from the control animals in this study compare to literature values?*

Even when the CC values are corrected, I am not aware of any studies measuring CC width at or around 10 and 22 days.

I don't know about the other brain measures at these ages.

*How might assessing the morphometry of brain structures in the rat at the ages chosen in this study (PND 10 and PND 22) affect interpretation of whether the findings are biologically significant?*

It will be necessary to obtain brain measures at least at one post-puberty age to get an estimate of a relatively stable adult brain. For example, the CC has many myelinated fibers, and myelination is not complete in the rat until at least 40 days of age. A group needs to be studied no earlier than 60-70 days to obtain a measure of a young mature brain.

**The next set of questions concerns biological significance.**

*Is there a 'pattern' to the observed changes that can be interpreted as a consistent effect on brain development...?*

The question of consistency of brain effects is sex-specific. In males, at both 10 and 22 days, perchlorate acts to increase brain measures; whereas in females at the same two ages, those exposed to the chemical *in utero* have smaller brain measures. It is likely both sets of effects are biologically "bad," and the findings suggest that the mechanisms of action may differ in the two sexes.

*There are several significant effect in males at the 1 mg/kg/d dose level that are not present at the higher doses...Is there an explanation for these observations of more regions with statistically significantly different measures at the lower doses than the high dose?*

Most biological functions will be U-shaped (or inverted-U shaped) over a wide enough range of an independent variable. There are several models that may apply. One model assumes that the body needs a certain minimum amount of the variable in question, up to an optimal, but beyond a certain point the variable can become toxic. Thus, the thyroid gland can be hypo, normal, or hyper.

Another model is that an insult has occurred to some bodily process (e.g., via a drug at a toxic level) and the body tries to compensate (structure enlarges) until it reaches a point of exhaustion (structure gets smaller as it moves toward collapse). This could fit the perchlorate findings.

However, it is not certain that a wide enough range of perchlorate doses has been built into the study to speak confidently about U-shaped functions.

*Is there a known or likely explanation for the apparent difference in direction of the change in brain morphometric measurements in males and females...?*

The most obvious answer is that the sex hormones are involved and that perchlorate is having different effects upon a testosterone background than upon an estrogen background.

With respect to the corpus callosum, there are interesting sex differences in the distribution of myelinated and unmyelinated fibers. We have found, in the genu of the rat, that females have a greater number of unmyelinated fibers than do males, while there is no difference in the number of myelinated axons (Mack et al., 1995); Juraska and Kopcik (1988), studying the splenium, reported similar findings. An EM study will be necessary in a future study.

*Are the statistically significant changes in linear dimensions observed in this study considered biologically significant? If not, what...would be...?*

As I indicated above, the prudent decision is to assume that any significant change related to perchlorate should be considered biologically significant until proven otherwise.

**The next set of questions address relationships with behavior**

*Are the statistically significant changes observed in linear dimensions of brain regions in pups likely to be associated with behavioral/functional effects in animals?*

For the corpus callosum, even when the proper measurements are made, it is most unlikely that the behaviors usually studied in the rat will be affected by callosal morphology. Balogh et al. (1999) studied this issue by testing mice from the 129 strain on a variety of behavioral tasks, and then determining the status of their corpora callosa. This strain of mouse is known to have callosal dysgenesis, with some mice having no callosum at all. Balogh et al. failed to find any relationship between the behavioral measures and callosal status. This is not surprising since it is rare to find a behavioral test given to rodents that requires the sharing or integration of information across both hemispheres. This is in contrast to primate research where interhemispheric tasks have been developed. This leads to the ironic conclusion that the CC findings (when done properly) may well have greater implications for the human corpus callosum than for rodent behavior.

*Have the appropriate behavioral endpoints been examined...?*

With the exception of the "M-maze," the other tasks are familiar ones in a behavioral laboratory. However, as noted earlier, the sampling of possible behaviors represented by these tests is very narrow. For example, no measures were obtained of spatial learning (see comment in next paragraph), non-spatial associative learning (e.g., discrimination learning), fear-based active learning (one-way or two-way shuttlebox), Pavlovian learning, novelty/exploratory behavior, or spatial and non-spatial working memory, to name a few important behavioral processes.

The M-maze is idiosyncratic. I have not heard of it before, and only found 4 references to it in Pub Med. It presumably is a measure of spatial learning though the maze can also be solved with a non-spatial strategy. I would have expected the Morris maze to be used to assess spatial learning.

**The last set of questions concerns relevance to humans**

*Would the brain effects observed in rats be expected to be observed in humans as well?*

If the corpus callosum, when properly measured, yields significant effects for the perchlorate treatment, this is likely to be found in humans as well.

*How might species differences in brain morphometry and development affect the appropriateness of the rat as a model for human neurodevelpment?*

This is a question to which there is no unequivocal answer. In some areas, there are strong relationships (e.g., the endocrinology of the rat, genetics of the mouse or fruit fly). In other arenas, the relationships are weak (e.g., social behavior). In general, where there is structural homology (such as found in neurotransmitters, hormones, and the CNS and ANS), researchers feel there is a reasonable likelihood that the rat is an appropriate model, though certainly not the only animal model. The classical example where the rat was not an appropriate model was with the drug thalidomide. Here the rabbit was found to model the human condition much more accurately.

With respect to development, the rat has the great advantage of allowing one to go from birth through puberty in 40 or so days with a life span around 3 years.

The bottom line is that the question of which rodent behaviors, structures, chemicals can be related to the human can only be determined empirically.

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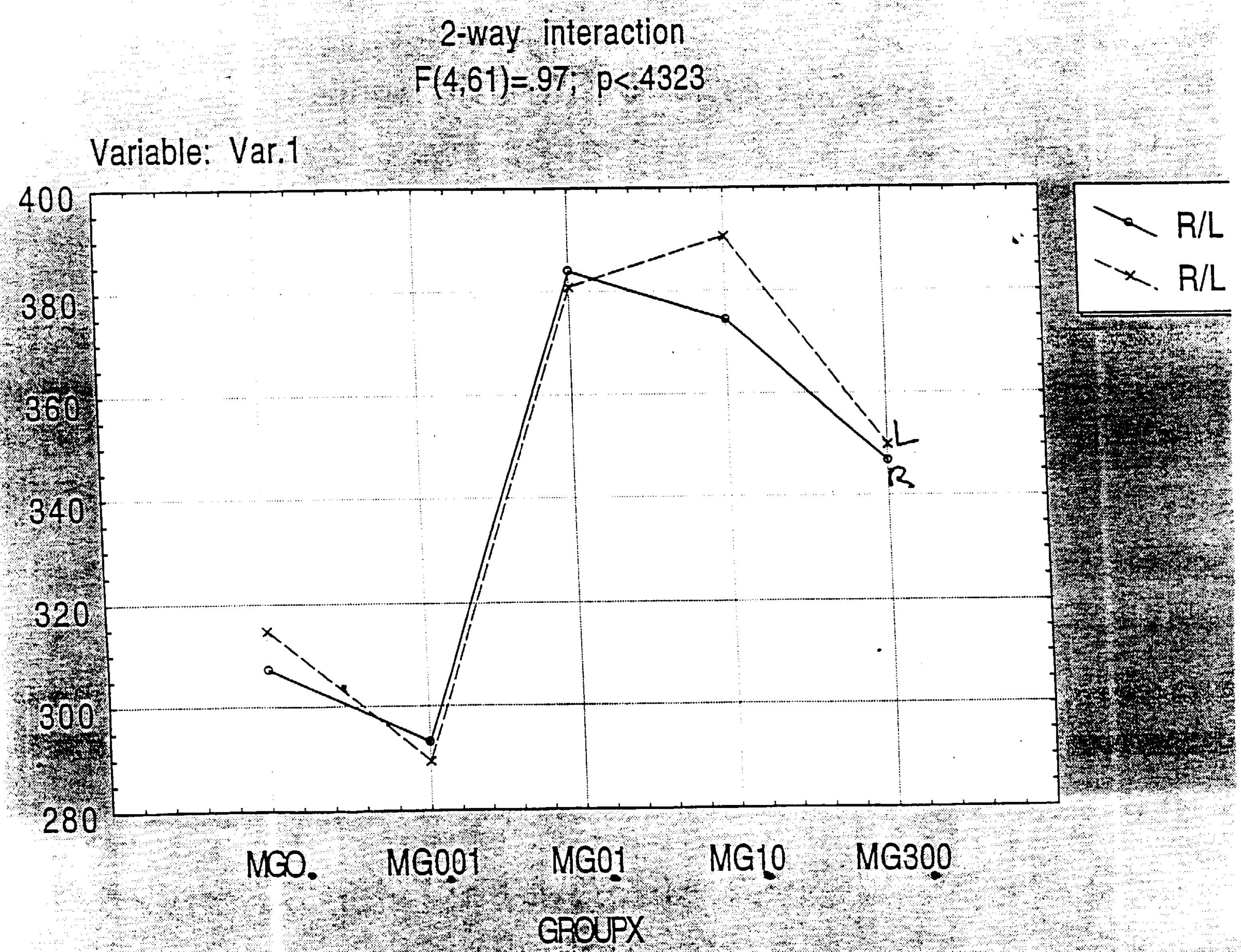
R/L CC PROFILE





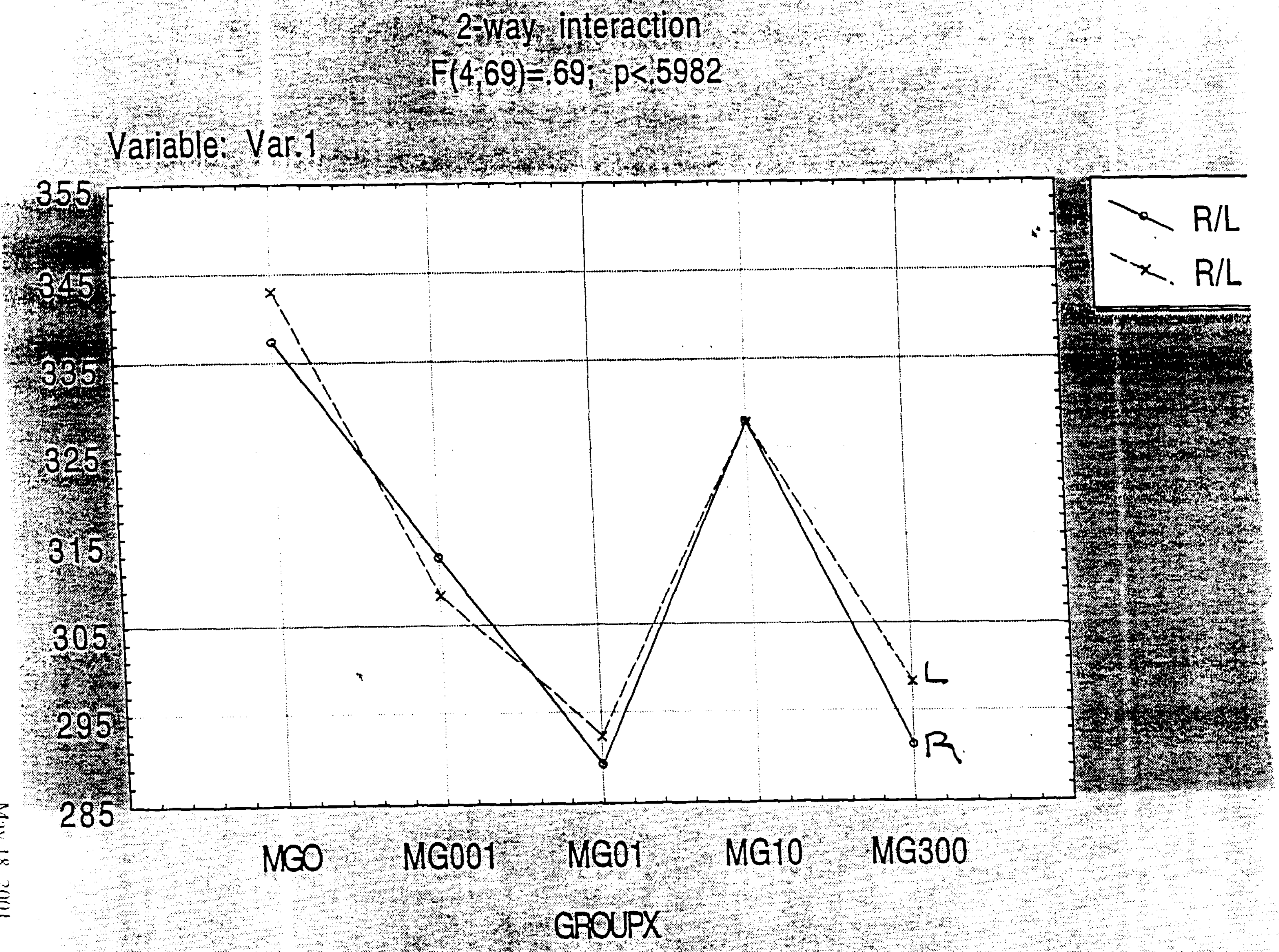

STATISTICA GENERAL MANOVA

summary of all effects; design: 1-GROUPX, 2-R/L

| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | [redacted] | 43636.58 | 61 | 5282.061 | 8.261279 | .0000216 |
| 2 | 1 | 503.43 | 61 | 473.182 | 1.063933 | .3063922 |
| 12 | 4 | 457.44 | 61 | 473.182 | .9667347 | .4323342 |

FIG. 1

DAY 10 FEMALES
R/L CC PROFILE




STATISTICA GENERAL MANOVA

Summary of all effects; design: 1-GROUPX, 2-R/L

| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | [redacted] | 13192.57 | 69 | 6510.078 | 2.026484 | .1002428 |
| 2 | 1 | 203.38 | 69 | 231.212 | .879617 | .3515779 |
| 12 | 4 | 160.62 | 69 | 231.212 | .694681 | .5981840 |

FIG 2

MALE PROFILE OF
AVERAGED R/L CC SCORES

| STATISTICA GENERAL MANOVA | LSD test; variable AVGMCC Probabilities for Post-hoc Tests MAIN EFFECT: GROUPX | | | | |
|---|---|---|---|---|---|
| GROUPX | {1} 312.8214 | {2} 291.3846 | {3} 382.6000 | {4} 382.4688 | {5} 348.4000 |
| MG0 {1} | [redacted] | .2761106 | .0014697 | .0003785 | .0633754 |
| MG001 {2} | .2761106 | | .0000647 | .0000096 | .0042089 |
| MG01 {3} | .0014697 | .0000647 | | .9948923 | .1031771 |
| MG10 {4} | .0003785 | .0000096 | .9948923 | | .0659637 |
| MG300 {5} | .0633754 | .0042089 | .1031771 | .0659637 | |

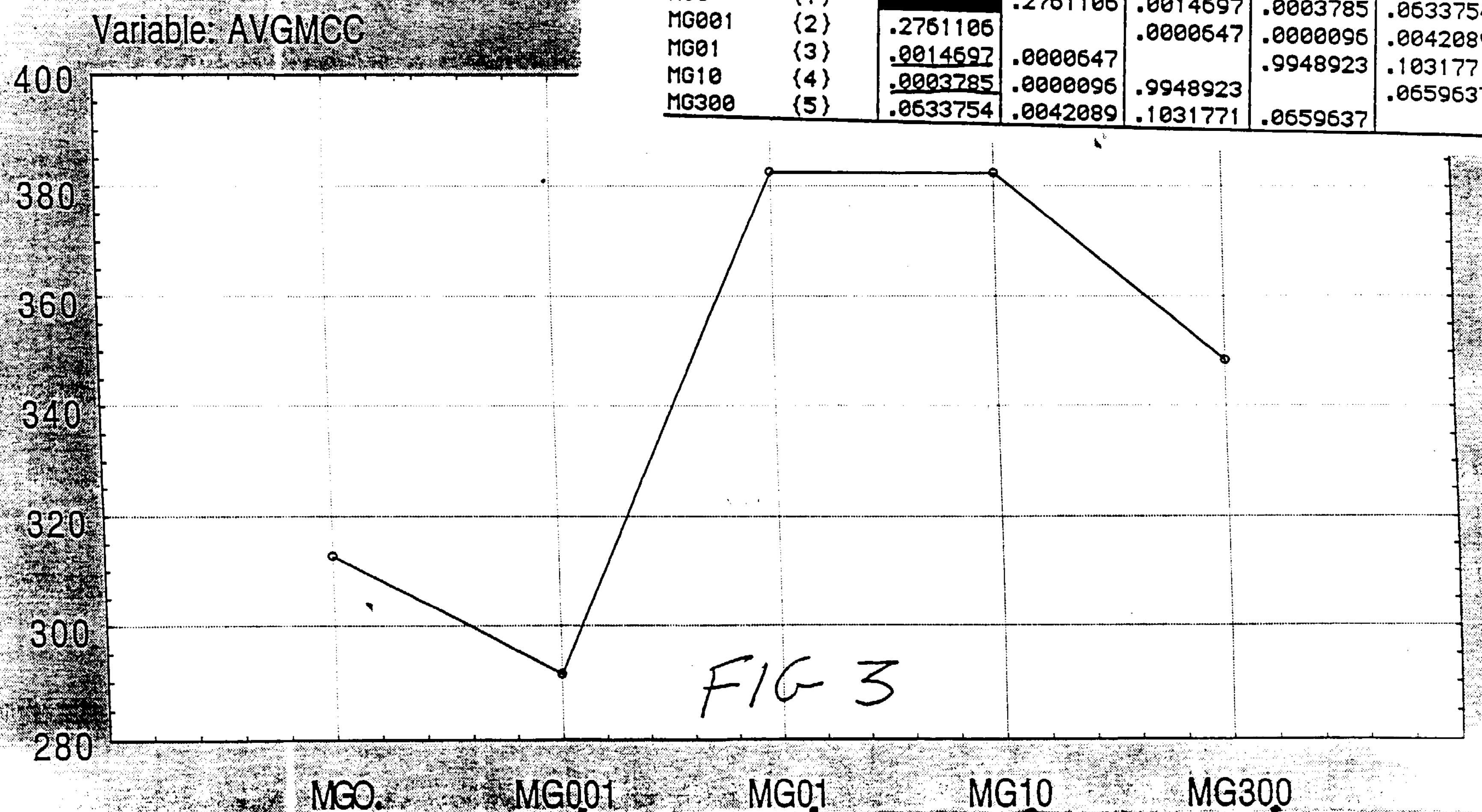



FIG 3

| STATISTICA GENERAL MANOVA | summary of all effects; design: 1-GROUPX | | | | | |
|---|---|---|---|---|---|---|
| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
| 1 | 4 | 22106.14 | 63 | 2566.406 | 8.613656 | [redacted] |

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[redacted] 10

FEMALE PROFILE OF
AVERAGED R/L CC SCORES

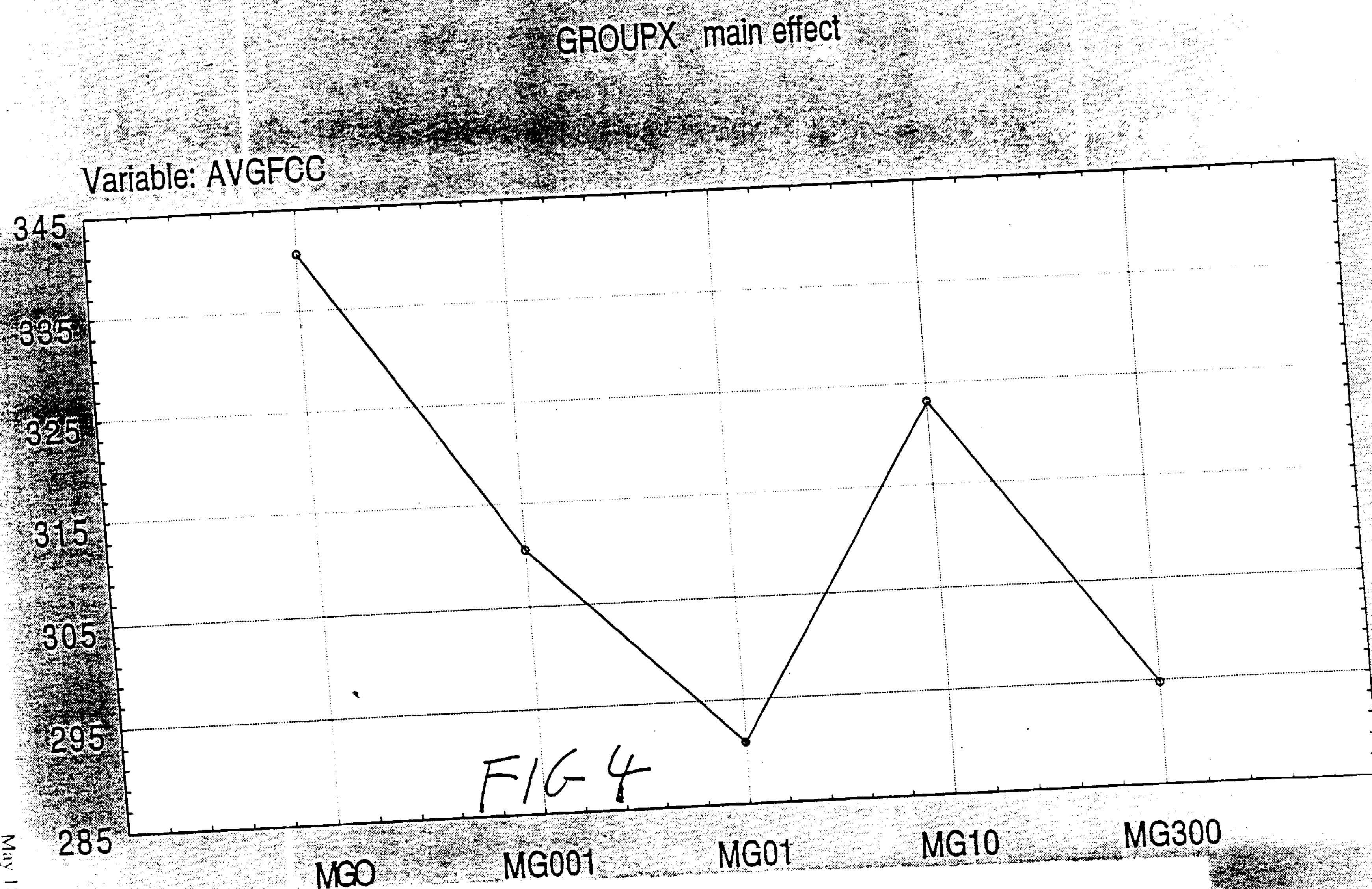
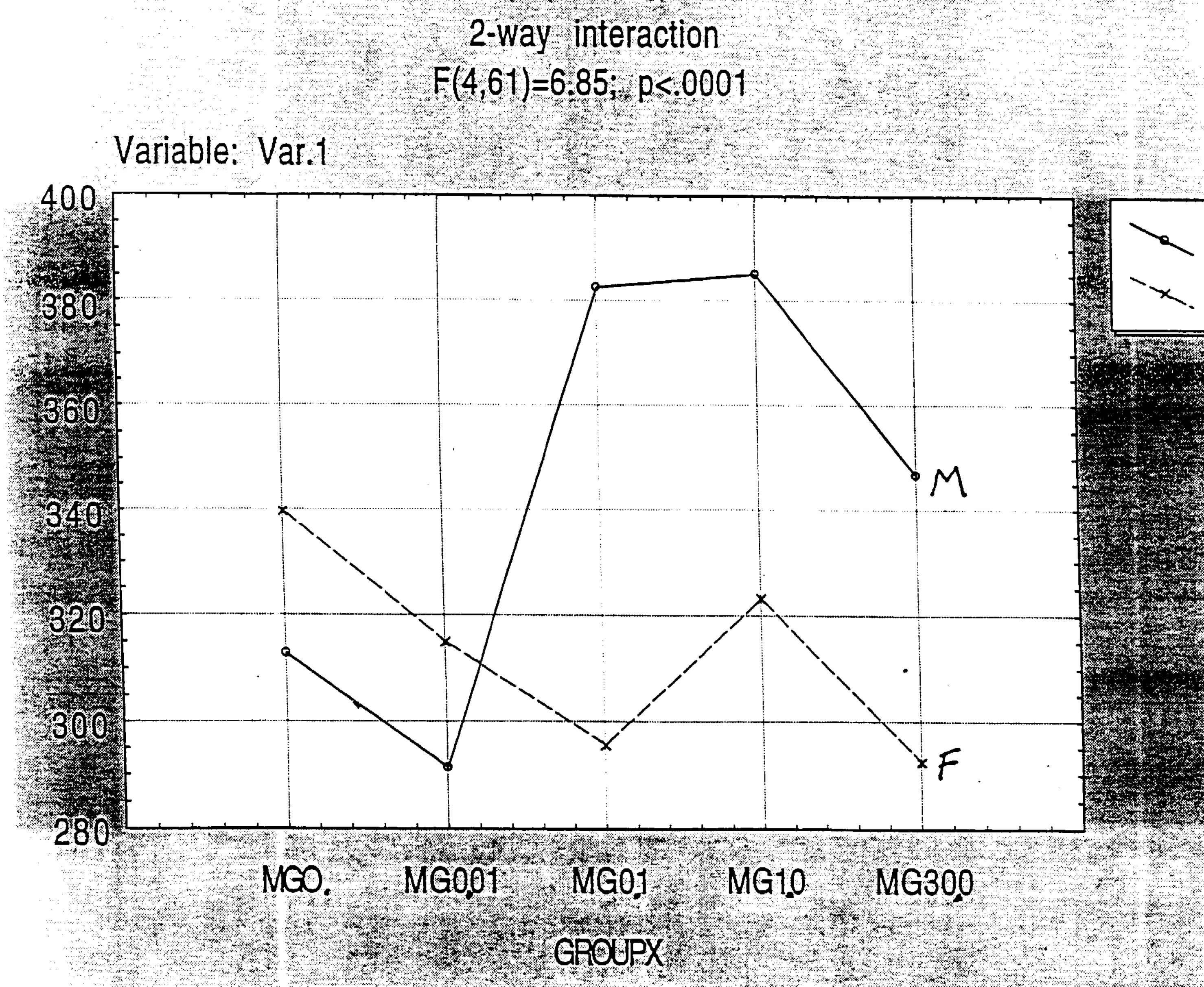



FIG 4

| STATISTICA GENERAL MANOVA | summary of all effects; design: 1-GROUPX | | | | | |
|---|---|---|---|---|---|---|
| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
| 1 | [redacted] | 6532.570 | 72 | 3165.083 | 2.063949 | .0944537 |

MALE/FEMALE PLOTS OF
AVERAGED R/L CC SCORES



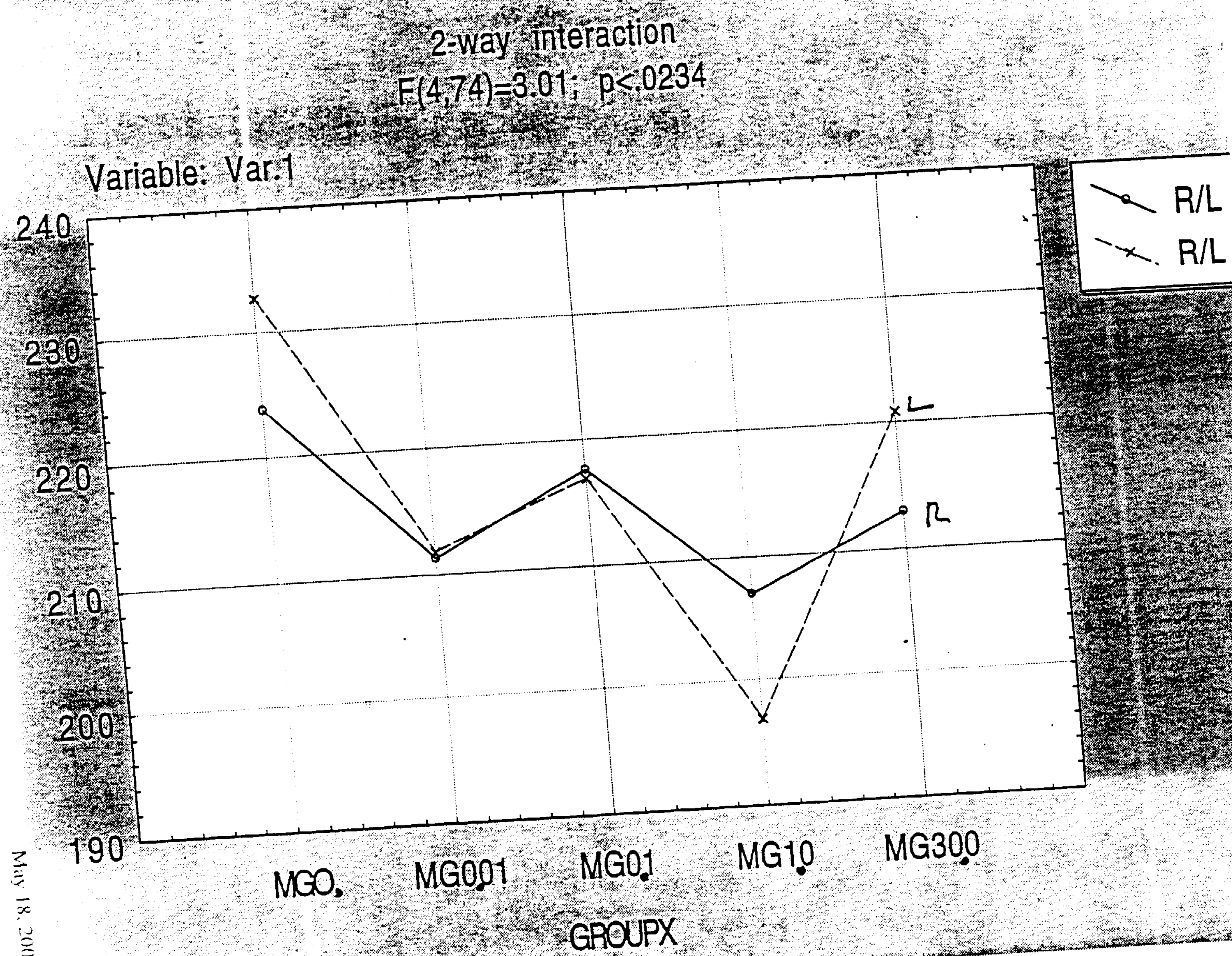

STATISTICA
GENERAL
MANOVA

Summary of all effects; design:
1-GROUPX, 2-M/F

| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | [redacted] | 10262.96 | 61 | 3329.114 | 3.08279 | .0223169 |
| 2 | 1 | 30240.87 | 61 | 2534.725 | 11.93063 | .0010100 |
| 12 | 4 | 17359.74 | 61 | 2534.725 | 6.84876 | .0001276 |

FIG 5

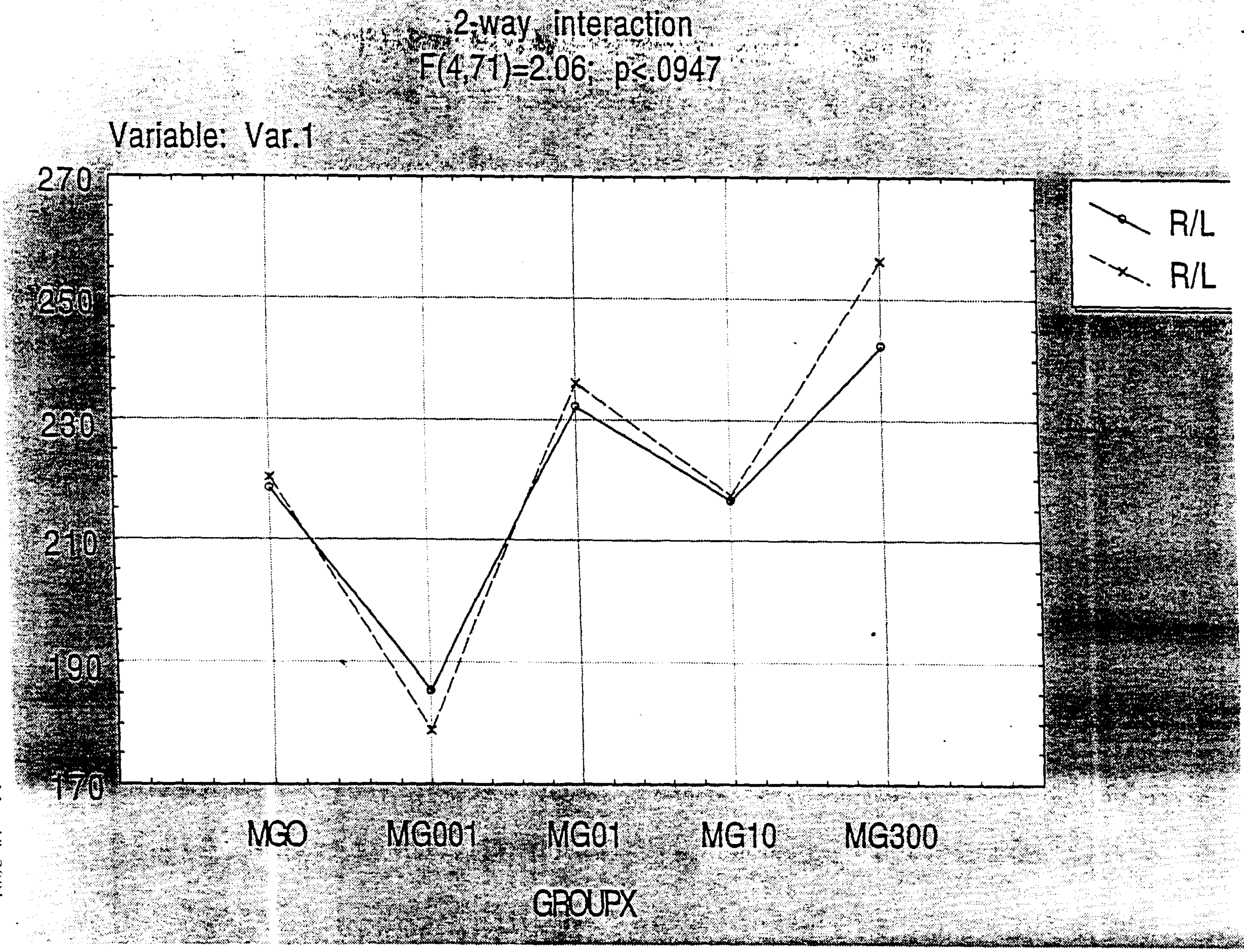
R/L CC PROFILE




| STATISTICA GENERAL MANOVA Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
| --- | --- | --- | --- | --- | --- | --- |
| summary of all effects; design: 1-GROUPX, 2-R/L | | | | | | |
| 1 | [redacted] | 2939.103 | 74 | 3364.255 | .873626 | .4839617 |
| | 1 | 65.773 | 74 | 158.872 | .413999 | .5219357 |
| | | [illegible] | 74 | 158.872 | 3.008678 | .0234061 |

FIG. 6

[redacted] TEMBERS
R/L CC PROFILE




STATISTICA GENERAL MANOVA

summary of all effects; design: 1-GROUPX, 2-R/L

| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | [redacted] | 18615.00 | 71 | 2765.558 | 6.731010 | .0001188 |
| 2 | 1 | 290.38 | 71 | 188.698 | 1.538868 | .2188702 |
| 12 | 4 | 309.41 | 71 | 188.698 | 2.063690 | .0946536 |

FIG. 7

MALE PROFILE OF
AVERAGED R/L CC SCORES

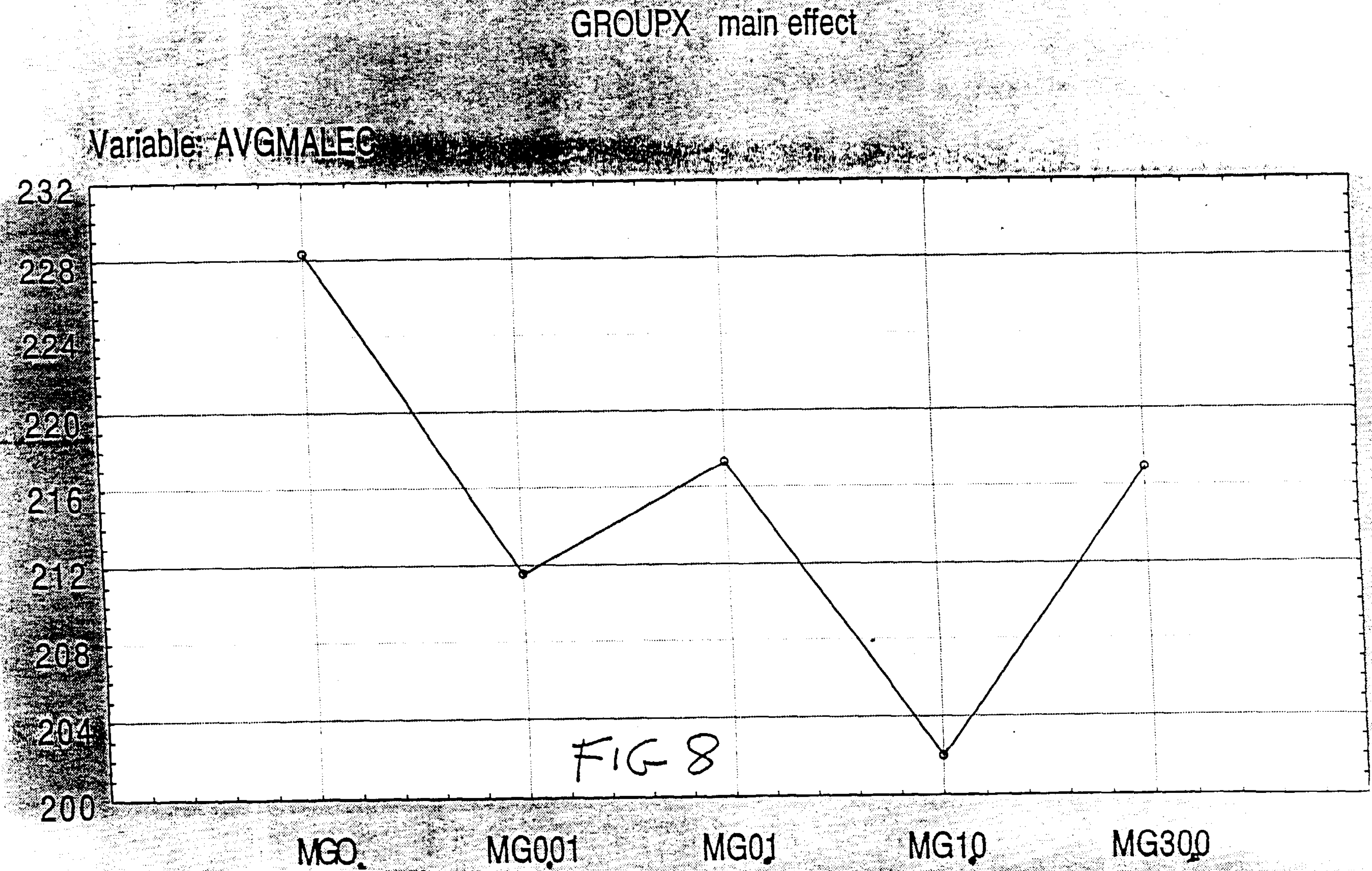



FIG 8

| STATISTICA GENERAL MANOVA | summary of all effects; design: 1-GROUPX | | | | | |
|---|---|---|---|---|---|---|
| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
| 1 | [redacted] | 1469.551 | 74 | 1682.128 | .8736265 | .4839617 |

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T.ERX DAY 22
FEMALE PROFILE OF
AVERAGED R/L CC SCORES

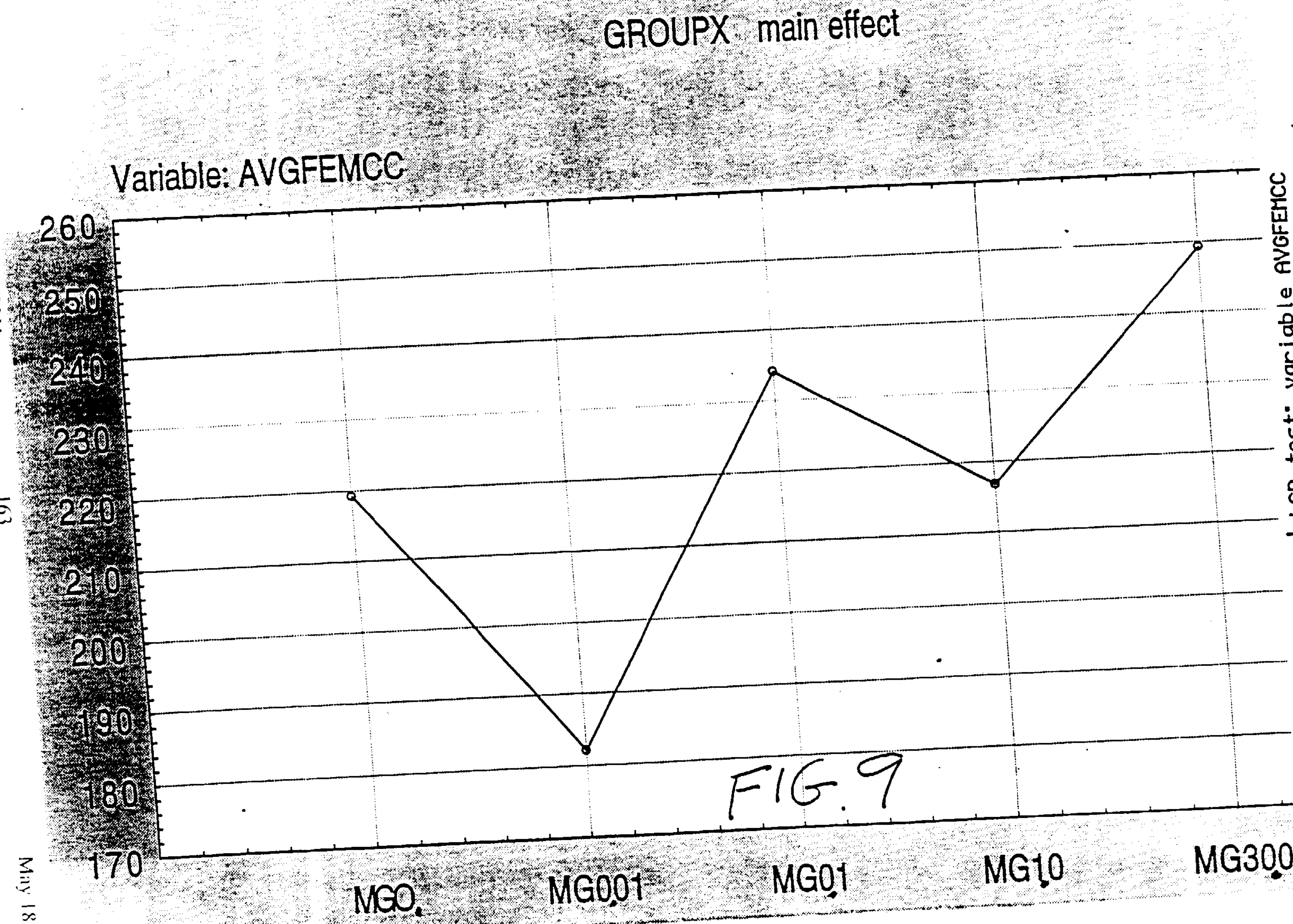



FIG. 9

STATISTICA GENERAL MANOVA

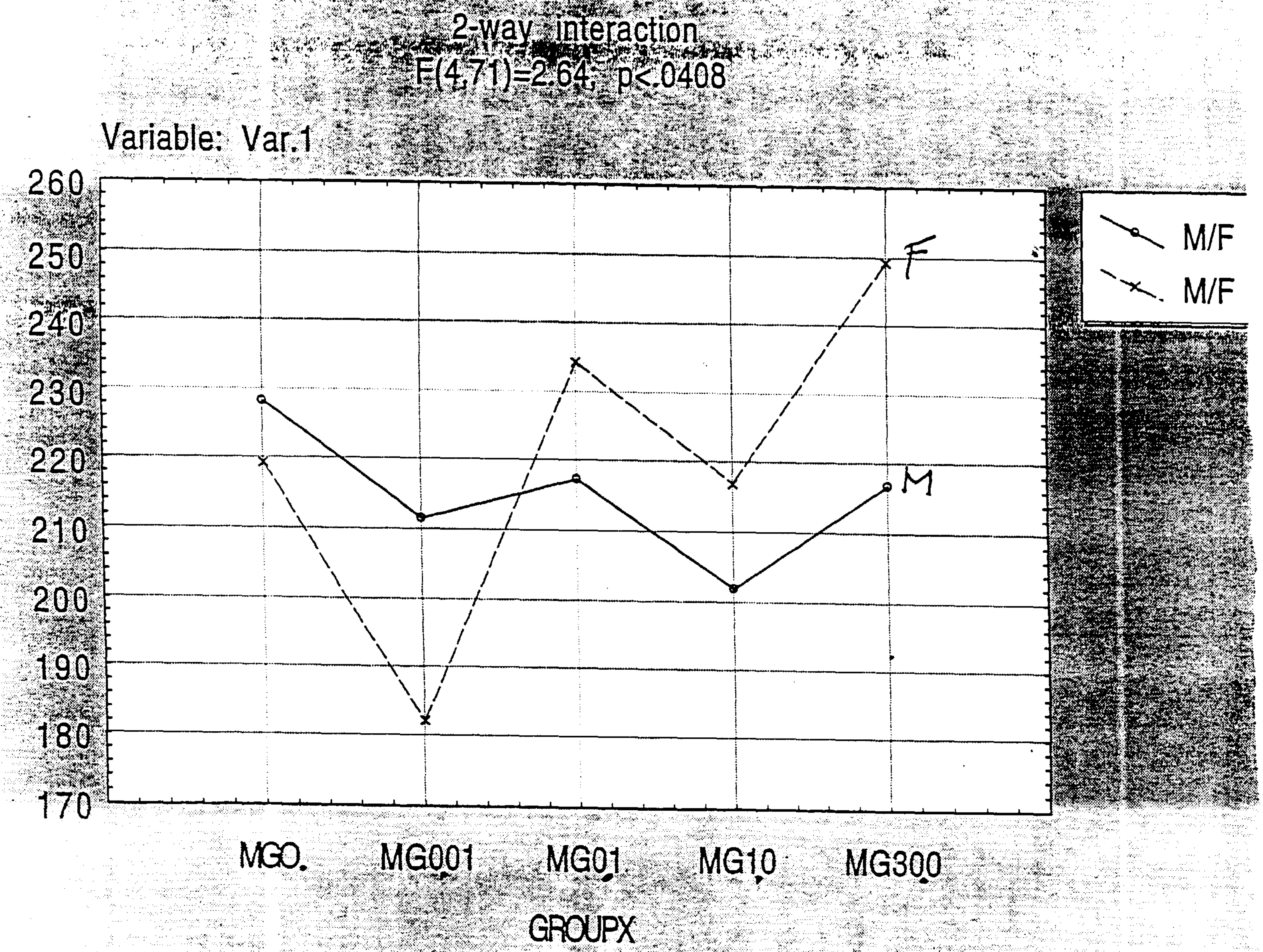
LSD test; variable AVGFEMCC
Probabilities for Post-hoc Tests
MAIN EFFECT: GROUPX

| GROUPX | | {1} 219.2813 | {2} 182.1562 | {3} 234.2667 | {4} 216.8750 | {5} 249.3077 |
|---|---|---|---|---|---|---|
| MG0 | {1} | | .0061545 | .2659434 | .8553004 | .0339481 |
| MG001 | {2} | .0061545 | | .0002166 | .0101645 | .0000074 |
| MG01 | {3} | .2659434 | .0002166 | | .1973503 | .2893938 |
| MG10 | {4} | .8553004 | .0101645 | .1973503 | | .0223296 |
| MG300 | {5} | .0339481 | .0000074 | .2893938 | .0223296 | |

STATISTICA GENERAL MANOVA — summary of all effects; design: 1-GROUPX

| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | | 9307.498 | 71 | 1382.779 | 6.731010 | .0001188 |

MALE/ FEMALE PROFILES OF
AVERAGED CC SCORES




STATISTICA GENERAL MANOVA

summary of all effects; design:
1-GROUPX, 2-M/F

| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | [redacted] | 6324.575 | 71 | 1444.816 | 4.377427 | .0032050 |
| 2 | 1 | 1018.821 | 71 | 1685.954 | .604300 | .4395248 |
| 12 | 4 | 4448.183 | 71 | 1685.954 | 2.638378 | .0408481 |



FIG. 10

Review of Primedica 2001
Janice M. Juraska
Mar. 18, 2001; revised Apr. 28, 2001

I am assuming that whoever reads this review, has also read Primedica 2001. Therefore, I am not summarizing the results except as needed for my comments. I also assume that the abbreviations (e.g., DL22 dams) are understood by the reader.

It is clear from Primedica 2001 that the effects of perchlorate do not dramatically alter neurodevelopment. However, the results of Primedica 2001 are inconclusive with regard to more subtle effects that are needed for the calculation of a Reference Dose of perchlorate. The levels of TSH in the treated dams were increased even at the lowest dose of percholorate, and there were signs of decreased T4 and T3 levels at the highest dose and often at lower doses. Likewise, pups at all of the sampled ages showed decreased levels of T4 or T3 at the highest doses (often at other doses as well), and male DL22 pups had decreased T4 levels at all doses. Despite hormonal changes, the neural effects do not correspond to the indications of decreased levels of thyroid hormone in the dams or in the pups. There are two explanations that are not mutually exclusive: 1) there are technical inadequacies to the present study; 2) the effects of perchlorate may be complex with different systems altered in different ways at various doses. It is impossible to deal with the second possibility until the problems of the first are addressed.

1) Technical problems

**The fixation of the brains**. The groups were not sectioned at the same time (see Neuropathology Report, Day 22 Postpartum Rats, p. 13). The control and the highest dose were sectioned and examined first and the other groups were added in order of dose. This could affect the size of various components of the brains. In the experience of my laboratory group, fixative slows the gross degeneration of the brain but cellular components do slowly break down. This is why anyone examining cellular structure with electron microscopy embeds the tissue within 24 hours of fixation by perfusion. We have found that the brain changes size with time in refrigerated fixative. First it shrinks for several weeks, then in subsequent weeks it swells. Time between fixation and sectioning must be held constant between the groups.

In the quantitative measurement of neural structures, it is standard practice to perfuse the animal through the cardiovascular system while the animal is under deep anesthesia. This ensures that fixation occurs quickly and simultaneously throughout the brain. Distortions can occur as the brain fixes from the outside in with immersion fixation. The time taken by diffusion with immersion fixation also allows degeneration of cellular components which makes the brains even more vulnerable to the shrinkage and swelling over time that was described above.

**Blindness.** It was also reported that not all measurements were performed "blind". This is not acceptable. The bias of the experimenter, often unintentional, cannot be allowed.

**The measures.** Linear measurements are not the most sensitive method for detecting alterations in neural structure. If there is a change, it does indicate that there have been effects. However, it is easy to miss effects when the measures are linear. Measuring volume would be a step in the right direction. For example, a 5% difference in the thickness of a particular cortical area may be difficult to detect due to inter-animal variability. If there is a 5 % difference in every dimension, then the resulting 14% difference in volume may be more easily found. Volume measurements are especially important for a structure such as the hippocampus because it changes in shape with each section. Consequently, the linear size will be very dependent on the section chosen for measurement. This is obvious in the wobble between the right and left sides of the hippocampus for the sections shown in this report.

The above is true even for the corpus callosum which can be represented in two dimensions as it crosses the midline. The measurements done here were not technically the corpus callosum but instead the cortical white matter. From my experience in examining this structure with electron microscopy (Juraska & Kopcik, 1989; Kim et al., 1996; Kim & Juraska, 1997), the tightly packed axons and glia seen at the midline become diluted with neurons and processes oriented in all directions once the pathway enters the brain. Hence, a midline measurement of the structure is essential. A mid-sagittal measurement would also be preferable to the coronal section. This should be done *en block* because it is virtually impossible to take a mid-sagittal section that holds together (see Kim et al., 1996 for the method). The area of the corpus callosum should be taken, not thickness. Area is more likely to be proportional to the cellular measures that are ultimately of interest - axon number and myelination. A structure might not vary in thickness but can still vary in area. The area of subportions such as the splenium (posterior fifth see Kim et al. 1996) can also be taken.

A minor point – the regression analysis was the simple graphing of average linear size versus dose. This did not reveal any new information. Why weren't individual animal means plotted against the individual animal's hormone level?

2) Complicated effects

**Cellular changes.** The ultimate question is not whether the size of various neural structures are affected by perchlorate but rather if structure is altered. Size is being used as a substitute for cellular and biochemical measures. There is a literature on the neuroanatomical effects of hypothyroidism (reviewed in Lauder & Krebs, 1986) that indicates that several complicated cellular processes are altered. Size measurements can be inconclusive because more than one cellular effect is occurring. For example in the corpus callosum, the number of axons may be increased (due to disruptions in axon withdrawal) while myelination may be decreased. Thus there might be no effect on size of the structure even though callosal function may be compromised. Such complicated effects make interpretations of size measurements difficult during development when several different cellular events are occurring asynchronously.

There is also the problem that DL22 is still a time of development for several structures in the rat brain. For example, we have found that axons continue to withdraw from the splenium of the corpus callosum after day 25 and myelination is just starting at day 25 (Kim et al., 1997). Neuron death (apoptosis) continues in the female visual cortex to at least day 25 (Nunez et al., in press), and hormonal manipulations after day 20 change the number of neurons in the adult visual cortex (Nunez et al., 2000a). There is also dendritic growth and regression between days 30 and 60 in the visual cortex (Juraska, 1982) and days 43 to adulthood in the somatosensory cortex (Wise et al., 1979). Likewise, synapses increase 1.5 fold between days 21 and 41 in the dentate gyrus of the hippocampus (Cowan et al., 1981).

With regard to quantification of the number of fibers in the corpus callosum, it should be noted that unmyelinated axons are not detectable with the light microscope. They are far too small for the resolution of light microscopy and require electron microscopy and excellent fixation to be detected. Myelin can be detected in the light microscopy at high power (>1000X). The sections have to thin (1 micron) for myelin to be discernable from background (see Nuñez et al., 2000b for method).

All this suggests that it would be useful to do an electron microscopic study of the corpus callosum in animals that were exposed to perchlorate during development. These rats should continue to be exposed through adulthood because this would model the situation that humans exposed to the substance would experience. It also may influence whether there is "catch up" from deficits occurring during development. This is especially relevant for the corpus callosum because my laboratory has recent evidence that myelination continues in this structure during adulthood (Nuñez et al., 2000b) making catch up a possibility.

**Recommendations**
The current study is inconclusive. Another study should be conducted in which animals are perfused with fixative and the time from perfusion to cutting is held constant for all subjects. Measurements should all be performed blind and be taken as volumes (area in the case of the corpus callosum). The above is standard practice in the scientific literature.
The experimental design should include adult rats that have been exposed to perchlorate their entire life to model the human condition. Cellular measures should be taken such as the cellular composition of the corpus callosum. The number of synapses in the hippocampus or cerebellum also are good candidates for investigation given the literature on hypothyroid effects.

Although I was not asked to comment on the behavioral studies done prior to Primedica 2001, I would like to add that none of the behaviors examined in Argus 1998 tapped either hippocampal or cerebellar function. All of the behavioral tasks in Argus 1998 were very simple. A radial arm maze test (baited and unbaited arms) for hippocampal function and a rotating rod for cerebellar function seem necessary.

Given the large sex differences in detectable effects to perchlorate, there is a possibility for interactions with the gonadal steroids. It would be useful to test reproductive abilities in rats that have been exposed to perchlorate all of their lives.

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**Review of Brain Morphometry Results from a Perchlorate Toxicity Study (Primedica 2001)**

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## Summary of Morphometric Data Review

Specific comments regarding the five issues noted in the guidelines are included below. A summary of conclusions follows. Linear measures of a variety of forebrain structures, solely in the coronal plane, demonstrate statistically significant (t-test), small changes in size at various exposure levels. A gender analysis reveals a trend towards increases in males, and decreases in females. Occasional side-effects (left versus right) are reported, but are probably due to technical caveats of using the coronal plane for matching anatomical level. The statistical analysis initially focused on a comparison of each treatment group (II-V) with the control group (I). While these comparisons revealed statistically significant changes at some levels of exposure, there is no clear dose-response effect with perchlorate exposure, making it very difficult to determine whether the data are biologically significant. The linear regression analysis, examining the interaction across groups, revealed a potentially complex relationship between levels of perchlorate exposure and changes in brain morphometry. The statistical tests used may not be adequate to resolve whether the relationships are biologically signicant, and it is strongly recommended that a statistician whose expertise extends to such biological systems is consulted. In the context of the data prsented in this report, the so-called 'bell-shaped' or inverted 'bell-shaped' response has parallels in certain biological systems, where it has been shown to be meaningful. The complex pattern of altered morphometry at various doses is in contrast to the reproducible effects of perchlorate on T3 and T4 levels at doses $\geq 1$ mg/kg-day. There is one potentially serious caveat in the experimental design (handling of pups during the first two weeks postnatally; noted below) and in the analysis of histological material (only coronal sections); both could affect the interpretation of the biological significance of the morphometric changes. No comments regarding the behavioral assessments are included, as this reviewer is not an expert in the field.

Overall, the morphometric data do reflect consistent effects of perchlorate on the structural development of select structures in the forebrain. In both males and females, the most potent dose for causing increases (males) or decreases (females) in the linear dimensions of certain structures <u>is not</u> the highest dose (30mg/kg-day), but rather 1mg/kg-day. In the context of understanding the link between perchlorate-induced decreases in TH levels, which are induced consistently at doses >1mg/kg-day, the data indicate that such TH alterations may be necessary, but clearly are not sufficient to cause the statistically significant changes in the size of specific brain structures. The data do not reflect what is typically seen in experimentally-induced hypothyroidism. It is more likely that the induced morphometric changes by perchlorate are due either to normal variation in the measure used in the Primedica study, or to complex effects on molecular characteristics that were not measured in this study. The degree to which the reported altered brain structure is biologically significant can only be hypothesized, because linear measures of changes in brain structures are not adequate to define structure-function relationships. In addition, the appropriate end point for morphological analysis was not included in the study (sexually mature rats), which is necessary to eliminate the possibility that the measured changes are simply due to a modest developmental delay. It is important to note here that in general, behavioral deficits and altered size of specific brain structures cannot be correlated directly. As an example, gross malformations, such as lissencephaly, hydrocephalus and pachygyra can result in severe mental retardation. These are all examples in which brain pathology is evident. Hydrocephalus, however, can be severe without necessarily leading to mental retardation. From the examination of the thumbnail images included in the report, there is no evidence of brain pathology in the perchlorate study. Very modest morphological changes, discerned by morphometric analyses, are reported in certain diseases of the human brain. For example, neuropathological analysis of brain sections or structural magnetic resonance imaging (MRI) report very modest changes in schizophrenia (cf. 10-18% decrease in the size of the hippocampus, thickness of prefrontal cortex), autism (8-12% decrease in amygdala volume, 8-12% increase in cerebellum volume, 10% increase in brain weight), and depression (see Lewis and Lieberman; Minshew et al, 2001 for reviews). Based on the findings here, the <u>magnitude</u> of consistent changes in the linear dimension of various forebrain structures are within the range of size changes observed in disorders of human brain function. However, in the context of whether the specific morphometric changes reported in the Primedica 2001 study are biologically (i.e. functionally) relevant, the linear morphometric analysis is inadequate to be interpreted in this fashion. Again, this is due to the inherent problem in linking structure-function relationships in the brain. As such, one cannot determine from the morphometric findings whether the consistent changes (at 1mg/ml) in corpus callosum, hippocampus and cortex thickness are adverse. The study design would need to be altered substantially to assess more relevant characteristics of brain organization to link perchlorate exposure with any potential behavioral deficits.

## Issue 1: Design of Primedica (2001)

*Please comment on aspects of the experimental design that would affect the interpretation of the brain morphometry studies.* There may be an important caveat in the design. Postnatal handling of pups and lactatating dams adds a confound that could affect brain development and maternal care. Meany and co-workers (Caldji et al, 2000 for review of data) have shown that limited handling (as little as 15 minutes of separation of pups from dam during the first postnatal week of life) is sufficient to alter gene expression (eg. corticotrophin releasing factor) and response to stress. Maternal care (licking) is altered significantly by the handling as well (Caldji et al, 2000 for review of experimental design). The recording of animal and dam weights resulted in handling during this period. While all groups were treated identically, the experimental design could have introduced a confound in which an interaction between perchlorate exposure and maternal care or neuroendocrine changes in the pups resulted in a complex biological effect. There is no data in the reports suggesting that perchlorate affects general pup weight or growth. Thus, a new study should eliminate handling effects by using parallel litters that are not assessed behaviorally or morphologically, but used instead to monitor weight.

The analysis of forebrain structures requires the use of material sectioned in the three cardinal planes. The design in the present study did not include such analysis, which presents a serious problem in data analysis and interpretation. The assessment of certain brain structures in the coronal plane is difficult (eg. length of corpus callosum; width of certain subfields of the hippocampus). The use of the coronoal plane only is likely to introduce inconsistencies due to section asymmetry and the inherent difficulties in matching rostro-caudal levels across brains between treatment groups. Side asymmetries were evident in the thumbnail images provided in the report, which is the most likely reason for the inexplicable side effects of some linear measure. They are not likely to be biologically significant.

*Were the morphometric methods used appropriate for detemining an effect on brain development?* Linear measures are a crude measure of biologically significant changes in brain structure and development. As a first attempt to examine the possibility of perchlorate-induced gross changes in brain structure, it is reasonable. The methods used here are not sufficiently sensitive to reveal clear structure-function relationships. Rather, cell-based assays, in which specific structural changes at the levels of the fundamental functional units, the neuron and glial cell, are much more sensitive measures of altered development (Levitt, 1998 for review of types of assays used in developmental studies), and it is strongly urged that such cell-based assays be incorporated into any future studies. However, a review of the literature shows that the use of such cell-based assays is not widespread in toxicology studies of brain development because such methods are not high throughput. Again, many examples of more sensitive assays are in the literature and methods and specific suggestions could be provided upon request if a lower throughput assessment of risk were to be pursued in the future.

*Is the sampling procedure used in this study for microscopic evaluations appropriate to look for an effect that is mediated through the thyroid hormones or TSH?* There are two issues here.

The design and implementation of the sampling procedures for doing linear measurements were done appropriately, although the single plane of section is a major caveat for analyzing some forebrain structures. However, this reviewer recommends against using linear measures to look for crude changes in the size of specific structures as a sensitive measure for documenting effects of thyroid hormones or TSH. Instead, the study design should have included sufficient power to allow for other quantitative methods of cell-based assays. For example, one can readily investigate the effects of perchlorate on proliferation (bromodeoxyuridine labeling) or cell death (TUNEL staining of histological sections. Both assays have been used in experimental systems in which levels of thyroid hormones or TSH have been manipulated (see Koibuchi and Chin, 2000 and Pasquini and Adamo, 1994 for reviews). Again, it should be stressed that these methods are low throughput, and are not be amenable to the study design here with multiple groups of perchlorate exposure, multiple brain regions, and multiple ages. The study design would need to be changed in order to pursue the more appropriate assays.

*Do measures made in this study have inherent sources of variation, and was the inherent variability adequately controlled in the study? How does the variability reported in this study compare with the expected variance?* All morphometric analyses have inherent sources of variation, but the variation in the means in the report of linear measures are within expectation for experimental error (except one area, see below). Variation can be introduced at a number of levels. Tissue processing in which dehydration is a component introduces inherent source of variation, due to differential shrinkage of brain structures. Based on the appearance of the sections in the thumbnails provided, it appears that the interbrain variation in shrinkage and sectioning quality is low, thus reflecting very well-controlled processing methods. There is variability across measures (+4-20%), and this is likely due to differential tissue shrinkage, operator error in making morphometric linear measurements across different brain samples, and inherent difficulties in matching anatomical location because of asymmetric sectioning in the coronal plane. The variability is generally ≤10%, which is routine for the experimental design used here (for example, see Guillèry and Herrup, 1998). The higher variability, particularly observed in measurements of the corpus callosum, is avoidable by using sections collected in the sagittal plane.

*Did the techniques used result in adequate homology in the brain sections and did they adequately control for sources of variation in the brain morphometric measurements?* The methods for tissue processing were well-controlled and resulted in the maximal homology of brain section quality. The issue of immersion versus transcardial perfusion can be debated. Immersion fixation used here does not provide the optimal cellular preservation, but introduced minimum variation in fixation quality. Transcardial perfusion, unless done by one individual (with internal controls for fixation quality), can introduce serious variations due to variability of fixation quality. In future designs, power analysis of the numbe of samples should be done to estimate the sample size needed to control for these technical variations. The coronoal plane of sectioning introduces greater variation in the measurement of specific areas (see below).

*How does the use of coronal sections affect the ability to compare the results with values reported in the literature?* All analysis was performed on sections cut in the coronal plane. This is adequate for analysis of cortex and striatum. This plane of section, however, introduces serious problems in matching anatomical level for analysis of corpus callosum and hippocampus (corpus callosum, see Bishop and Wahlsten D, 1999). The former is best evaluated in the sagittal plane, the latter in the horizontal plane (Burrows et al, 2000). Cerebellum is best analyzed in the sagittal plane.

*How do the linear dimensions of measured brain regions from the control animals in this study compare to literature values?* Linear measures provided are comparable to values in the literature (Bayer and Altman, 1991 for comparative sectioned material at P10 rat).

*How might assessing the morphometry of brain structures in the rat at the ages chosen in this study (PND 10 and PND 22) affect interpretation of whether the findings are biologically significant?* Analysis of two pre-weaning ages provides an opportunity to assess the <u>rate</u> and <u>degree</u> of change of the size of particular brain structures. The analysis <u>does not provide</u> an opportunity to determine if any of the observed differences are due to differences in the rate of developmental changes (so-called delays or enhancements) that contribute to specific measures of brain or structure size. These differences may eventually normalize post-puberty.

## Issue 2: Biological Significance of Results

*Is there a 'pattern' to the observed changes that can be interpreted as a consistent effect on brain development, based on the results from the Primedica (2001) study and the Argus (1998) neurobehavioral study?* Several consistent patterns were observed regarding brain development. It is important to emphasize that while there may be patterns to what was assessed here, these measures are not amenable to direct cause-and-effect evaluation with the neurobehavioral study.

The following conclusions may be amended with additional statistical analysis. If changes were measured in a particular structure, they tended to reveal increased size in males, in contrast to increased or decreased size in females. Consistent changes in the linear size of structures that contribute to the corpus callosum were measured. It is noted here that the corpus callosum measures included the hippocampal commissure (anterior commissure level); thus, the increases (or decreases) in hippocampal subfields that contribute to the hippocampal commissure are consistent with this interpretation. Significant morphological effects in males and females tended to occur at intermediate doses (1mg/ml), rather than at the highest doses. Statistical analysis should be done on the original data in order to determine whether this pattern is stochastic or a real biological phenomenon.

*There are several significant effects in males at the 1 mg/kg/d dose that are not present at the higher dose (e.g., left* *striatum and right CA1). Also, there are several parameters that are affected by dose, but that show a similar magnitude of effect over the very large dose (e.g., cerebellum) range in this study. Is there an explanation for these observations of more regions with statistically significantly different measures at the lower doses than the high dose?* Biologically effective doses of growth factors can show very similar phenomenon. Thus, intermediate doses of a particular agent may produce a more substantial biological response than low or high doses (or recent examples, see Koshimura et al, 2000; Satou et al, 2000). For example, hepatocyte growth factor (HGF) serves as a stimulator of cell movement. At subthreshold doses (<1ng/ml), cells do not move. At intermediate concentrations (5-10ng/ml), cells are stimulated to move. At high concentrations (>50ng/ml), cells do not move(Powell et al, 2001). Response of neurons in culture to glutamate at low doses (1um) is negligible, robust at intermediate doses (10-30um), and absent at high doses (100um). A dose-response curve appears similar in shape to the effects measured in males and females for the following regions:

At P10 -

Right CA1, Right Hipp, Left & Right Corpus Call, Left Striatum, Right Parietal Cortex, Left and Right Frontal Cortex

At P22 -

Cerebellum

A dose-response curve appears similar in shape to the effects measured in males was seen in females for the following regions:

At P10 -

Left & Right Corpus Call, Right Frontal Cortex

At P22 -

Cerebellum

An inverted bell-shaped curve was obtained for most affected changes in females, where size differences were reported as decreases.

At P10 -

Right & Left CA1, Right and Left Hipp, Right and Left Striatum

*Is there a known or likely explanation for the apparent difference in direction of the change in brain morphometric measurements in males and females (e.g., increases in males, decreases in females)?* The short answer is no, there is no known explaination. There is however, a possible explanation that would require direct testing. Thyroid hormone regulates gene transcription. Estrogen regulates gene transcription as well. It is possible that the differences in males and females measured here is a consequence of differential gene regulation in males compared to females, perhaps due to an additional regulatory element that has great activity in developing female brain. Literature searches did not identify studies examining effects of TH on genes also mediated by estrogen.

A second explanation involves the experimental confound of handling that was noted above. It is possible that the handling experienced by the litters had different effects on males and females, thus introducing a confound in one sex but not the other. There is evidence that such early postnatal handling can alter stress hormone responsiveness in females, but not males (see Caldji et al, 2000, for review).

*Are the statistically significant changes in linear dimensions observed in this study considered biologically significant? If not, what degree and/or direction of change (i.e., 10% increase, 20% decrease etc.) would be considered biologically significant?* Well-respected clinical studies do not use linear measures to discern biologically significant alterations in brain development. The comments that follow may be applicable here only if one assumes a linear relationship between the linear measures and volume and/or area changes in the same structures. This was not determined in the present study, so unfortunately, there is no direct way to compare. To reiterate, a major deficiency in the Primedica study is the fact that there is not a linear relationship between change in the size of brain structures and the qualitative or quantitative extent of functional deficits.

One can hypothesize about the extent of size change that would be needed to correlate with seriouis brain dysfunction. As noted above, volume and areal changes in brain structures in the 10-20% range, which are statistically significant, also can be biologically significant. As one example, the brain weight of children with autism, at autopsy, is approximately 10$ larger (Minshew et al, 2001 for review of data). Cerebellar size is increased to a similar extent (Minshew et al, 2001). In adults with autism, size differences are no longer seen, although the characteristic functional deficits remain (Minshew et al, 2001). In another example, the increase in ventricular size in schizophrenia is 8-12%, with a similar decrease in hippocampal volume (Lewis and Lieberman, 2000 for review of data). The extent of these changes is independent of age at which the measures were made.

## Issue 3: Biological Plausibility of Results (consistency with mechanism of action)

*Are the changes observed in linear dimensions of brain regions consistent with changes in the thyroid hormone or thyroid histopathology observed in the same groups of animals?*

Consistent changes in corpus callosum, cerebral cortex and hippocampus occur in the 1mg/ml treatment group, which did result in decreased thyroid hormone levels. The 30mg/ml dose, which also caused a reduction in thyroid hormone, did not result in altered linear measures. Thus, there is not a linear relationship between decreased thyroid hormone and the size of brain structure that were measured. As noted above, it possible that thryoid hormone reduction is necessary, but it cannot be sufficient to cause the linear decreases (males) or increases (females).

Studies cited below report that altered thyroid hormone (TH) levels can have very different effects on brain populations and the genes that regulate the development of specific brain regions. It is important to note that thyroid hormone levels in different developing brain areas vary greatly (Joffe et al, 1994). Studies of the role of thyroid hormones have focused almost exclusively on hypothyroid state and its effects on cerebellar development (341 references in a PubMedline search using thyroid hormone and cerebellar development as keywords; 3 references using thyroid hormone and brain development as key words). The reason for this is the relative ease of analyzing disruption of cerebellar morphology and the ease of causing the hypothyroid state. It should be pointed out here that the summary provided to this reviewer by TERA regarding T3 and T4 levels cites increased levels of hormone in males and females, at higher perchlorate exposure (p. 8 of report). Careful reading of the actual reports from Latha Narayanan revealed decreases or no change.

While many of the studies on the effects of TH on brain development have focused almost exclusively on the cerebellum (which was not changed consistently in the Primedica study), a modest number of studies have included data on the anatomical and cellular specificity with which TH acts during development. The literature search failed to reveal definitive studies on differential gender effects of TH reduction on brain development, though it should be noted that many developmentally relevant genes contain estrogen response elements. If certain genes were co-regulated by estrogen and TH, these could be susceptible to different effects of TH deprivation in males versus females.

TH will promote expression of specific genes, which if abnormally expressed, may result in aberrant brain development, with functional consequences. Genes encoding myelin basic protein (MBP), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and reelin are just a few examples of important developmentally related genes that have thyroid hormone response elements. Studies have been published using hypothyroidism as a model to demonstrate altered expression of these genes, with resulting changes in cell proliferation, migration, neuronal and glial differentiation. As noted above, the studies typically focus on structural effects in cerebellar development, where there are well-known reports of altered proliferation of cells, increased apoptosis, hypomyelination and delayed synapse formation (Koibuchi and Chin, 2000; Pasquani and Adamo, 1994, for review of literature).

There are some reports that are relevant to the effects seen on select forebrain structures. Tera et al (1998) note that astorcytes isolated from the cerebral hemispheres are more responsive to TH regulation of gene expression than astrocytes from the brainstem or cerebellum. This is consistent with the findings of more consistent size changes in the forebrain structures compared to the cerebellum in the Primedica 2001 study. Pambo et al (1998) report that the MAL proteolipid protein gene, expressed by oligodendrocytes, is regulated by TH during development, but not in the adult. Altered gene expression by oligodendrocyted could alter myelination and affect corpus callosum size. Garcia-Fernandez et al (1997) report very complex, dichotomous changes of an important protein in a state of experimentally induced hypothyroidism. They note brain region-specific and cell type-specific effects of hypothyroid state on the expression of lipocalin-type prostaglandin D2 synthase. This enzyme is responsible for prostaglandin D2 synthesis. Cajal-Retzius neurons, responsible in part for the proper migration of cerebral cortical and hippocampal neurons, exhibit reduced levels of the synthase in low Th state. In contrast, prostaglandin D2 synthase protein levels were higher in neurons of the CA1 and CA3 regions and the dentate gyrus of the hippocampus. The findings highlight the very real situation in which reduced levels of TH can have varied effects on the state of different target populations in the developing brain. Unless measured directly, there is no way to predict accurately whether the expression of a particular gene or protein will increase or decrease in the hypothyroid state.

*Do the results reported for the structures measured correspond to effects observed in published studies of severe hypothyroidism?* No. Hypothyroidism results in developmental delays, reduction in size of cerebellum due to altered cell proliferation and cell death and reduced myelination (see Koibuchi and Chin, 2000 for review). None of these changes are conistently seen in the Primedica study. Several of the studies cited above report specific effects on genes expressed in the forebrain, in particular, the cerebral cortex and hippocampus. These changes at the molecular level could be extrapolated to effects on developing brain structure, but this is hypothetical. Literature searches failed to identify studies in which states of hypothyroidism were induced during development and measurements were performed on the same forebrain regions showing changes in the Primedica 2001 study.

*Does the pattern of statistically significant changes observed in the brains correspond to the pattern that would be expected if these changes were being mediated by alterations in thyroid hormone levels?* As noted above, there are studies of effects of TH on genes expressed during development that could alter the developmental course of oligodendrocytes, astrocytes and neurons, but the design of the Primedica study does not allow this to be determined. The gender effects cannot be supported by any data in the literature that this reviewer could find. While perhaps surprising, it is important to note that the same abnormal level of thyroid hormone can cause increases or decreases in gene and protein expression in the same brain, but in different areas. Thus, it is not surprisign that the literature contains data that are consistent with the observed brain changes.

The most effective doses of perchlorate that induce thyroid hormone deficiences overlap with, but are not identical to those concentrations that resulted in reproducible changes in brain structure. The failure of the highest dose of perchlorate to induce structural changes, in the context of reproducible alterations in TH levels, indicates that if the changes in brain structures are biologically relevant, there must be other interacting elements driving the changes. The effects cannot be due solely to alterations in TH levels. As noted above, altered TH levels induced by perchlorate exposure may be necessary, but are not sufficient to cause the statistically significant changes the size of specific brain structures.

## Issue 4: Relationship with Behavioral/Functional Effects

*Are the statistically significant changes observed in linear dimensions of brain regions in pups likely to be associated with behavioral/functional effects in adult animals?* The design of the Primedica study does not allow this to be anawered unequivocally. However, as noted above, no studies with which this reviewer is familiar have determined a well-defined correlation between the extent of changes in the linear dimensions of the size of certain brain structures and functional effects in adult animals. It is possible, but it is more likely that an underlying cellular change would be responsible for any behavioral deficits. What may be considered very minor changes in size, for example, in autism, may reflect considerably more functional deficits than larger changes seen in hydrocephaly. The clear conclusion that there is no correlation reflects the inability of linear measures to be linked directly to a specific brain function. Changes in linear measures are caused by alterations in cell structure or organization. Again, any attempt to define a relationship between putative behavioral changes and structure would need to include the assessment of the specific cell populations and circuits responsible for mediating a particular function.

*Have the appropriate behavioral endpoints been examined in order to evaluate an effect of perchlorate exposure on behavior?* Yes.

**Issue 5: Relevance to Humans**

*Can you comment on whether the brain effects observed in rats would be expected to be observed in humans as well? If changes in linear dimensions of brain regions were to be observed in humans, would these changes result in a functional or behavioral deficit?* While there are many well-conserved events of brain development that are regulated by the same genes and proteins across species, the effects of perturbing the milieu in which the brain develops may or may not result in the same changes across species. It is possible that the brain effects of perchlorate in rats could occur in humans as well. It also is possible that it may not have any effect on brain size.

The first question under Issue 4 addressed the issue of the relationship between alterations in the linear dimensions of brain structures and functional or behavioral deficits. To reiterate, this reviewer does not know of any studies showing such a link, and would only conclude that there is a link if cell-based assays were used to show consistent changes at the same doses in which behavioral changes are induced.

*In particular, how might species differences in brain morphometry and development affect the appropriateness of the rat as a model for human neurodevelopment?* In the opinion of this reviewer, the differences in brain morphometry between lissencephalic and gyrencephalic animals is unlikely to be a major factor in using the rat as a model for human neurodevelopment. Thousands of neurodevelopmental studies have been done in rodents and the mechanisms that underlie specific neurodevelopmental events are well-conserved across species (see Levitt, 1998 for a brief review). A more likely confound in using the rat as a model of human neurodevelopment are the inherent differences in maternal care that are known to affect brain development (see above) and the possible differences in pherchlorate metabolism that may exist between rodents and humans. This reviewer is unfamiliar with the literature on the comparison of perchlorate metabolism and TH effects in different species.

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# REVIEW OF

# PROTOCOL 1416-003

entitled

**Hormone, Thyroid and Neurohistological Effects of Oral (Drinking Water) Exposure fo Ammonium Perchlorate in Pregnant and Lactating Rats and in Fetuses and Nursing Pups Exposed to Ammonium Perchlorate During Gestation or via Maternal Milk**

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## Background

The current studies and those completed earlier (Argus, protocol 1613-002) were designed to assess the public health risk of the presence of perchlorate in drinking water. Perchlorate has been found in drinking waters in several states, the levels generally ranging between 4 and 18µg/L. Perchlorate has in the past been used in the treatment of hyperthyroidism at doses exceeding 1gm/day, though it is generally no longer used due to significant negative side-effects and the development of better therapeutic modalities, such as the thionamide drugs and radioiodine ablation. Perchlorate is an anion that blocks active uptake of iodide, a vital component of the thyroid hormones, thyroxine (T4) and triiodothyronine (T3). It acts by competitive inhibition of the Na/iodide symporter in the follicular cell membrane of the thyroid follicles (Dai, Levy et al. 1996; Wolf 1998) leading to a deficiency in the production of thyroid hormone.

Hypothyroidism, the result of insufficient levels of thyroid hormone leads in newborn children and laboratory animals to lack of growth and in the extreme, a cretinous state, including poor motor, intellectual and behavioral development due to deficient maturation of the central nervous (Schwartz 1983; Legrand 1986; Oppenheimer and Schwartz 1997). Thus, it is of importance to properly evaluate the risk of the perchlorate content of drinking water supplies.

Several studies have been published to date attempting to evaluate the risk of the perchlorate to those exposed to it in industries responsible for the contamination of ground waters and to those drinking the waters. In neither circumstance have any adverse effects been noted. Lamm et al (Lamm, Braverman et al. 1999) evaluated workers at an ammonium perchlorate production facility in Nevada exposed on average to 1, 4, 11 and 34mg perchlorate per day. No differences among these groups were found in plasma thyroid hormone levels, thyrotropin (TSH) or plasma hormone binding, anti-thyroid peroxidase antibodies or on general physical examination. These workers estimated that the population around Las Vegas, an area with waters found to have about 18ppb perchlorate, would ingest only about 36µg perchlorate per day in an average intake of 2 liters of water per day, orders of magnitude lower than the control group in their study. Gibbs et al (Gibbs, Ahmad et al. 1998) reported a similar study in workers inhaling up to 436µg/kg with the same results. No perchlorate-related effect on TSH, T4 or T3 was demonstrated. Further, standard blood testing indicated there were no effects on liver, bone-marrow or kidney function. Lamm and Doemland (Lamm and Doemland 1999) found no evidence of increased risk of congenital hypothyroidism among about 700,000 births in those counties of California and Nevada with detectable perchlorate levels in the drinking water. Li et al (Li, Li et al. 2000) also found no differences in neonatal T4 levels in Las Vegas, where perchlorate levels are up to 15µg/L as compared to a control population in Reno, Nevada. The California Department of Health Services (California 1997) similarly reported no increase in the rate of congenital hypothyroidism in areas of that state in which measureable levels of perchlorate were found in the drinking waters. Lastly, Siglin et al (Siglin, Mattie et al. 2000) reported a study of perchlorate in the drinking water of rats using a range of doses from 0.01 to

10mg/kg/day. They found alterations in plasma TSH, T4 and T3 at all dose levels but thyroid histology was altered only at the highest dose. No other physiologic or biologic changes related to thyroid hormone action were noted suggesting the rats were not hypothyroid.

## Introduction

Since the primary effect of perchlorate is to block synthesis of thyroid hormones, the first question to be asked of these studies is, Were any of the doses of perchlorate used in these studies sufficient to induce a state of hypothyroidism? Clearly, these doses were sufficient to affect hormonal synthesis, although at best, the changes in plasma hormone levels observed were modest. In dams, fetuses and pups, at all times and in all treatment groups, TSH tended to be raised although these changes did not always reach significance. Similarly, plasma T4 and T3 levels were only modestly decreased even in those groups that showed statistically significant changes but group means tended always lower. Generally, as expected, the higher the intake of perchlorate, the greater the changes noted in plasma hormone levels.

These, at best, modest changes were generally reflected in the thyroid glands. Increased hyperplasia and hypertrophy and decreased colloid were only seen in the highest dose groups. The thyroid is well known to respond to deficiency in iodine intake with increased rates of hormonal synthesis and an increase in the ratio of T3 to T4 that is secreted before there is a detectable goiter or increase in hypertrophy and hyperplasia (Taurog 1996). Thus, plasma T4 may be more consistently reduced than T3.

Against expectations, in the previous study (Argus B-2), T4 and T3 levels were found to be increased in the perchlorate treated dams at PND 10 at the same time that TSH was increased. No attempt was made to explain these discrepant results in a manner consistent with the known biology of the thyroid hormones and their regulation by TSH.

Were these animals, in fact, hypothyroid? I believe the answer is clearly, NO. It is well known that hypothyroidism leads to a reduction in the growth rate of both dams, fetuses and pups, measured as change in body weight (Bonet and Herrera 1988; Sood, Schwartz et al. 1996; Schwartz, Ross et al. 1997). Pregnant dams show a réduction in weight gain within 2 weeks of the onset of hypothyroidism (Bonet and Herrera 1988) and a reduced growth rate is already evident in fetuses at G21 when hypothyroidism is established early in gestation (Schwartz, Ross et al. 1997). Although brain weight is also generally found to be réduced in hypothyroid pups, the degree of fall is less than that of body weight so that the brain/body weight ratio is found to increase (Legrand 1986). There was no difference in these parameters among any of the treatment groups at any time point. Added evidence against hypothyroidism in the dams is the lack of an effect on food intake. Hypothyroid adult rats generally reduce their food intake 20% or more (Westenend, Schroder-van der Elst et al. 1993; Syed, Thompson et al. 1999). Further, litter size is consistently reduced by as much as 50% in hypothyroid dams (Morreale de Escobar, Pastor et al. 1985). It was also noted in the earlier study (Argus) that, at the doses used, behavioral development was unaffected. As far back as the studies of Eayrs and colleagues (reviewed in (Schwartz and Oppenheimer 1979)), it was noted that in hypothyroid pups there was retardation of learning and of development of behaviors such as the righting reflex and the startle response.

Given the very modest changes in plasma hormone levels, it would be expected that brain T3 levels would not be altered in any significant fashion. T3 in brain is mostly a product of local conversion from T4 (Leonard and Koehrle 1996). It requires a drastic reduction in the amount of plasma T4 substrate entering the brain to significantly lower the T3 levels. The local deiodinase type II increases its activity in the attempt to maintain the normal T3 concentration. It would have been of value to measure such targets of thyroid hormone action as myelin basic protein and mRNA or Pcp-2 mRNA (Oppenheimer, Schwartz et al. 1996), deiodinase activity in brain (Leonard and Koehrle 1996) or hepatic malic enzyme activity or mRNA (Oppenheimer, Schwartz et al. 1996). These are accepted markers for thyroidal state.

Current therapeutic use of perchlorate is generally limited to patients who have developed hyperthyroidism as a result of treatment with the iodine-containing drug amioderone for cardiac arrythmias (Martino, Aghini-Lombardi et al. 1986; Reichert and de Rooy 1989) Administration of 1gm of potassium perchlorate per day is sufficient to block thyroidal uptake of iodine and increase its release from the thyroid gland allowing a return to normal function. Thus, in a 70kg individual the dose of perchlorate is approximately 14mg/kg/day, half the highest dose used in these studies. In my laboratory and in the literature generally (Fernandez Rodriguez, Galera Davidson et al. 1991; Sandhofer, Forrest et al. 1996), the concentration of potassium perchlorate used to induce hypothyroidism in laboratory animals is 1-2% (10 to 20mg/ml) in the drinking water. In a rat weighing 250gm drinking about 30ml per day this is a dose of 1.2 to 2.4gm/kg/day as much as 80-fold the maximum 30mgkg/day dose used in the current study. It is not clear on what basis the doses of perchlorate used in this study were chosen, since even the lowest dose, 10μg/kg/day is at least 20-fold higher than the estimated intake of a 70kg individual (see discussion above) in the affected areas.

## Neurohistology

Thyroid hormones act via nuclear receptors and the character of the dose response as it relates to receptor occupancy by hormone has been described (Coulombe, Schwartz et al. 1978). Responses, no matter the target, enzyme activity or mRNA, rise as fractional occupancy of receptors increases and a maximum response is observed with full occupancy. No sex related differences in the biology of the molecular mechanism of action are known nor have the effects of altered thyroid state been shown to be sex dependent.

There are a number of disturbing findings in this section of the report that suggest the unreliability of these data. Among them are [1] inconsistency of results with those in the previous (Argus) study; [2] a lack of dose-responsiveness; [3] differences in response between males and females, and instances of left/right sided differences in response; and [4] inconsistency with the relevant literature in this area.

[1] The reduction in brain weight in the perchlorate groups seen in the earlier study was not duplicated in the current round at any dose. Again, the increase in size at PND12 in the cerebellum earlier seen at 3mg was not seen at any dose in this study. In the earlier study, an increase in the size of the corpus callosum was seen at 10mg but not at the 3mg dose at PND12. At PND82, males showed an increase at 10mg, the females a decrease at the same dose. In the current study, an increase was seen at 0.1mg and 1.0mg but not 30mg in males at PND10, no change was evident in the females. Of interest, the coefficient of variation for the 10mg group in the earlier study was no greater than those observed in the groups in this study despite mixing the data for the sexes. If there truly were a difference of response in the sexes, the measure of variance should have been much larger.

[2] A lack of dose-response is evident throughout these studies both for any given brain segment and among brain segments. Although, generally plasma hormones were lowest in the 30mg dose group, most often this group showed no treatment effects. Nor is there consistency of the effects among doses from one study to another or, in the current study, from one brain segment to another. Changes are found at the very lowest doses but no other or for the intermediate doses but not the highest.

[3] I know of no data in the literature going back 50 years related to the actions of these hormones in brain that describes a difference in the biology of the thyroid hormones between the sexes or of differences in response from one side of the brain to the other.

[4] The literature in general demonstrates that in the rat, the postnatal development of the brain is most acutely dependent on thyroid hormone (Schwartz 1983; Legrand 1986; Oppenheimer and Schwartz 1997). One of the most well described effects of hypothyroidism in the newborn pup is the delayed development of the cerebellum. In the euthyroid rat pup, the external granular layer disappears by PND24. In hypothyroid pups the width of the external granular layer is greater than in euthyroid pups. This is due to a reduced rate of migration of the granule cells away from the external to the internal granular layer. Yet no difference in the cerebellar external granular layer was seen in this study.

Gravel et al (Gravel and Hawkes 1990; Gravel, Sasseville et al. 1990) reported that by PND25 the cross-sectional area of the corpus callosum was reduced in hypothyroid pups by almost half and that myelin content was less than 95% of control. No difference from normal was evident at PND10. Those authors did not indicate a sex related difference. The current results show an inconsistent increase in size in males and essentially no changes in the female pups.

## Conclusions

The doses used in these studies were sufficient to cause modest changes in the thyroid hormone economy. This is reflected in the generally observed increases in TSH and altered histology of the thyroid at the highest doses. The less consistent changes in plasma T4 and T3 are likely due to compensatory increases in thyroid hormone synthesis and secretion. This is also reflected in the increased hypertrophy and hyperplasia as well as the reduced follicular colloid at the higher doses.

The data, however, suggest that the intake of perchlorate was not sufficient to induce a hypothyroid state even at the 30mg dose. Unfortunately, no accepted tests of thyroid state were done to test this issue. Thus, it is concluded that any changes observed in the brains of these animals are likely not the result of reduced tissue T3 concentrations. It would have been of value to have measured the tissue T3 concentrations.

It is a serious weakness of these studies that the literature on thyroid/brain relations appears to have been ignored in choosing the endpoints to be measured. An enormous effort was given to these measurements. But there is no foundation in the literature suggesting that the size of most of these areas is affected by hypothyroidism. Further, none of the well-defined endpoints of thyroid hormone affect in brain, target gene mRNAs, myelin content, enzyme activities, etc that have been so well described over a fifty year period was included as control.

Because endpoints not known to be affected by altered thyroid state were used in this study, it would also have been of great importance to have included a positive control for the hypothyroid state and its consequences both peripherally and in brain. Methimazole, 0.025% in the drinking water, is routinely used as the drug of choice in studies such as these. It induces severe hypothyroidism in the dam and, because it crosses the placenta and is secreted in the milk, in the fetus and pups (Schwartz, Ross et al. 1997). It is quite effective when begun at 12 to 14 days of gestation prior to the onset fetal thyroidal synthesis of hormone at about day 15.

A final technical point. Many of the biochemical and molecular effects of hypothyroidism on brain are transient, first appearing at about 10 days of age and returning toward normal by 20 to 30 days of life ((Schwartz 1983; Oppenheimer and Schwartz 1997) and papers reviewed therein). It is, therefore, vital in future studies to be aware of the details of the time course of the effect of thyroid hormone on any particular target in order to choose the appropriate age at which to make the measurements.

Given what appear to be major weaknesses in the neurohistological data, including lack of dose response, sex dependent differences and lack of reproducibility, it is not possible to conclude there is any effect of perchlorate at these doses on brain development.

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MusWare Technology Inc.

# Review of Brain Morphometry Results from a Perchlorate Toxicity Study (Primedica 2001)

**April 11, 2001**

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## Introduction

This report is organized according to issues and questions posed in a document from TERA dated March 5, 2001, that gives background information on the perchlorate toxicity study and guidelines for the reviewers. The introduction provides a brief summary of the scope and methodology of the study.

Possible adverse effects of perchlorate on brain development were assessed by administering four doses and a control solution to pregnant and lactating rats in their drinking water, beginning two weeks prior to conception and continuing until termination of the study. A variety of measures of the fetuses and neonates conceived in those dams was assessed at four ages ranging from shortly before birth to a few days prior to the usual age for weaning. The measures of principal interest involved thyroid anatomy and biochemistry plus brain morphometry. Generally speaking, increasing doses of perchlorate increased the serum levels of thyroid stimulating hormone (TSH) and decreased levels of the thyroid hormones $T_4$ and especially $T_3$. There appears to be no question about the effects on the thyroid, and the Primedica study confirmed established knowledge in this respect. Effects on brain morphometry were less clear and consistent, and there is doubt about how strong conclusions from these data ought to be.

The design of the study is summarized in Table 1. At the outset of the study, there were 16 dams and their litters in each group of rats assessed at gestation age 21 days (DG21), lactation day 10 (DL10), and lactation day 22 (DL22). The DL10 and DL22 litters were culled to four male and four female pups on lactation day 5 (DL5), and some of the spare pups were sacrificed and assessed at that time. Thus, the DL5 data are not completely independent in a statistical sense from those at DL10 and DL22; whereas DL10 and DL22 data are taken from entirely separate litters of rats, the DL5 rats were littermates of some in the DL10 and DL22 groups. Thyroid and brain samples were evaluated for one male and one female from each litter, where littermates were in effect paired observations. For a number of reasons, the final sample size was less than 16 in several groups.

**Table 1 Study design**; dose in mg/Kg/day administered to dam; age when offspring were tested (Gray boxes indicate no brain morphometric data were available to the reviewer.)

| Age Dose: | 0 | 0.01 | 0.1 | 1.0 | 30.0 |
|---|---|---|---|---|---|
| DG21 | | | | | |
| DL5 | | | | | |
| DL10 | 14♂, 16♀ | 14♂, 16♀ | 11♂, 14♀ | 16♂, 15♀ | 15♂, 15♀ |
| DL22 | 16♂, 16♀ | 16♂, 16♀ | 15♂, 15♀ | 16♂, 16♀ | 16♂, 16♀ |

Results from the different ages were presented and analysed separately, but a great deal of the material in each age-specific section of the report is identical, which confirms that all ages were part of the same study. Cohabitation of the dam and sire began on January 10 or 14, 2000, for all groups, and all treatments and tissue collection were done at Argus Research Labs in

Horsham, Pennsylvania. Certain tissue samples were then shipped to other labs for histological evaluation and statistical analysis.

For the DG21 fetuses, perchlorate clearly increased TSH and decreased $T_3$ levels. No neurohistology has been done on these fetuses, however (p. 48). For DL5 neonates, perchlorate increased thyroid size of males by a small amount but had little or no effect on female size. No histology on the brains at DL5 has been done (p. 55). Thus, the only observations on brains presented in the report are at DL10 and DL22, and my assessment deals primarily with these data.

**Issue 1: Design of Primedica (2001)**

**A. Adequacy of the experimental design**

The animal husbandry and methods of administering the substances were conventional and appeared to be adequate.

The experimental design was a conventional dose-response study with independent groups at each of four doses plus a zero-dose control. Because the experiment was run as a single study, the design is a two-way factorial, the factors being dose and neonate age, with sex being a within-group factor. Because data were considered separately for males and females, it is apparent that the authors viewed this as a three-way factorial study, the factors being dose, age, and sex. Nevertheless, they conducted separate statistical analyses on four subsets of groups - DL10 males, DL10 females, DL22 males, and DL22 females. **This piecemeal approach does not allow a proper statistical evaluation of sex or age differences in treatment effects.** Although the design itself was adequate, a statistical analysis more appropriate to the design could have been employed. Analysis of a factorial design as separate subgroups reduces the statistical power of the many tests of significance and precludes a formal test of sex-by-dose interaction [47].

Another important aspect of the design is the choice of sample size. Sixteen rats per group is quite adequate for testing the average or main effect of dose, age or sex difference in cases where a moderate effect size is expected, but it is usually insufficient for testing the hypothesis that the factors act independently and do not interact. The crucial determinant of sufficient sample size is the magnitude of a treatment effect that the investigators would like to be able to detect [10, 11, 46, 47]. Whereas I as a reviewer am asked to assess whether a biologically significant effect is present (Issue 2), the authors themselves do not state at the outset how large an effect must be to be considered biologically significant. **Lacking a standard for a noteworthy effect of a potential toxin, it is not possible to make an informed choice of sample size for an experiment.** My educated guess is that the sample size employed was sufficient for ascertaining whether a significant linear log(dose) treatment effect of perchlorate was present, but the sample size was probably inadequate for judging whether males and females were differentially affected, or whether perchlorate effects were larger for DL10 than DL22 neonates.

Design includes the ages chosen for assessment of possible effects on the brain. My principal concern in this regard is that only immature rats were studied. Although it is generally recognized that decelerated growth of the rat brains continues for many months, there is no widely accepted age when an animal becomes "adult." Nevertheless, it is apparent that all major anatomical features of the rat brain are present at the age of reproductive maturity, after which changes in size of brain structures are generally quite small. The DL22 rats examined in this study had not yet been weaned and were far from being mature. Consequently, **it is possible that treatment effects of perchlorate altered the rate of development but had no influence on the eventual, mature size of brain structures.** Figure 1 portrays this problem. An additional group of older rats, perhaps animals 100 days after birth, would greatly enhance my confidence in conclusions about perchlorate effects on brain morphometry.

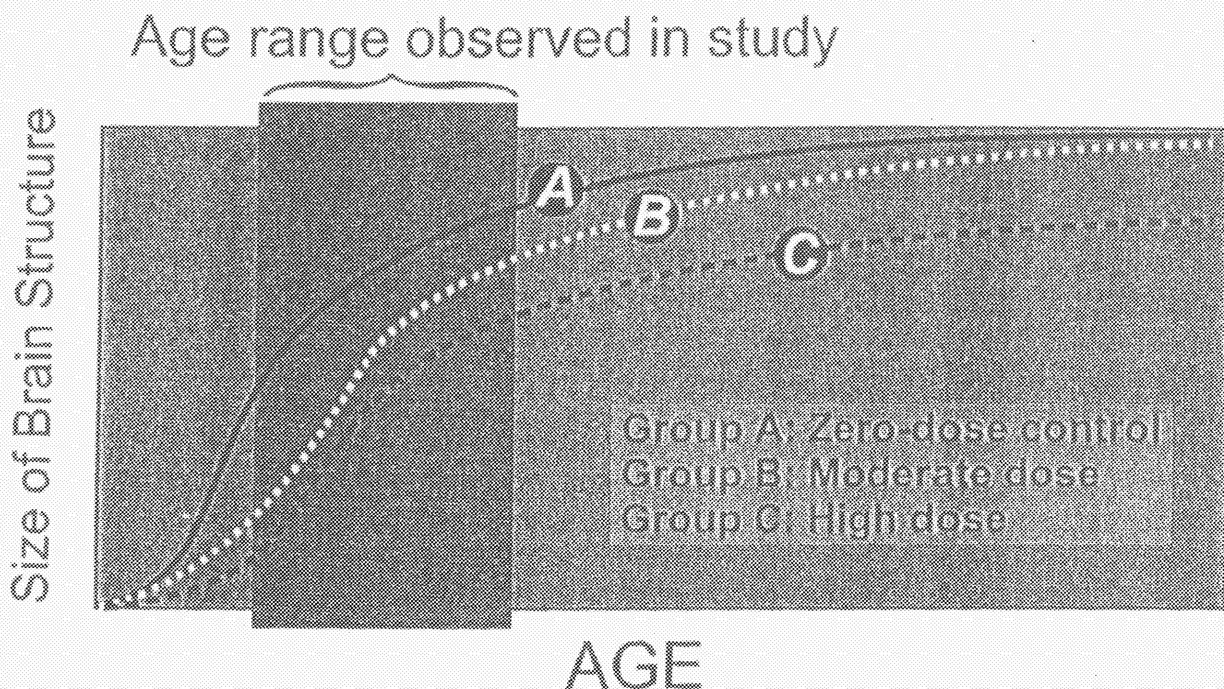


**Figure 1** Study of ages when brain is changing rapidly may not generalize to adult.

In addition to the question of long term consequences of perchlorate for adult brain structure, there is concern about a possible retardation of development. It is possible that a toxin might substantially delay myelination or the migration of granule cells from the external granular layer (EGL) of the cerebellum, for example. Thickness of the EGL is a particularly good indicator of developmental rate in certain age ranges because it disappears completely by a certain age, whereas most other quantities increase with age to an asymptote that is usually difficult to ascertain [41]. We have found that thickness of the EGL is useful for detecting the relative degree of retardation of brain development produced by a genetic variable, inbreeding [42], as well as undernutrition [53]. In order to serve as a sensitive indicator, however, **EGL thickness should be measured at an age that is midway between the age of its peak size and the first age when it disappears altogether.** Rats at DL22 would likely be near the age when the EGL normally disappears and therefore would be able to reveal only major, grossly abnormal retardation.

When assessing rate of development, it is also important to keep in mind that genetic strains tend to differ in the ages at which various developmental landmarks such as disappearance of the EGL are reached [42]. It would therefore be **desirable to evaluate perchlorate effects on more than one strain in order to establish the generality of dose-response.** In mice, there is a particularly large developmental delay imposed by inbreeding in embryos [52] and neonates [42]. The level of inbreeding in humans is generally very low, and it makes sense to study effects on non-inbred stocks of rats, but **it would be helpful to know the degree of genetic variation present in animals chosen for a study of substance safety.**

One further aspect of design warrants brief comment. The person making morphometric measures of brain tissue should always be "blind" with respect to treatment, sex and even age of the animals being assessed. **It is not clear that an acceptable level of blind assessment was achieved in the Primedica study**. Age would have been difficult to conceal in a study with only two ages where brain sizes were very different. Perchlorate dose and sex could easily be concealed from the observer, however, especially because different labs did the tissue collection and the histology. For the DL10 rat brains evaluated by Consultants in Veterinary Pathology Inc., it is stated on page 7 of the neuropathology report that three dose groups were studied "in a non-blinded fashion" and only the zero dose controls and highest (30 mg/Kg/day) dose group were measured blind. This is a very peculiar procedure and is clearly inadequate. Looking closely at Tables 1 (males) and 2 (females) in that report, where individual scores are shown, it is seen that Rat Number is identical for males and females within a dose group. Normally, a coded rat number written on a slide or vial of brain tissue is all that is needed to render morphometric assessment blind, but I am not sure just what was done in this study. Did the observer know the sex of the rat brain tissue being measured? I cannot tell from the report. The DL22 rat heads were sent to Experimental Pathology Laboratories Inc., but I could find no statement whatsoever about the degree of "blindness" achieved in making morphometric measurments. The report merely states on page 8 that all measures were taken "by the study pathologist." **It is essential that we be told what information about dose and sex was available to the pathologist at the time the measurements were done.** As with the DL10 rats, "Rat Numbers" in Tables 1 and 2 are identical for males and females, so I suspect that those were litter numbers rather than rat numbers.

## B. Appropriateness of morphometric methods

Brain tissue from DL10 and DL22 rats was fixed by immersion in 10% neutral buffered formalin, a method that is inferior to intracardiac perfusion but is nevertheless adequate and is much quicker. The brain was then sectioned into slabs that were embedded in paraffin prior to slicing for histological study. Paraffin embedding entails passage of the tissue from water to alcohol to toluene or xylene and then to paraffin wax. This common procedure usually shrinks the tissue by a very large amount, reducing linear measures by almost 40% of their true values in some tissue [44, 51]. This reality of the histology lab is not a major cause for concern, provided the investigators take care to determine the degree of shrinkage and then correct their measures to restore them to valid figures. In the Primedica study, however, **thickness measures were not corrected for shrinkage of the tissue during histological processing, and no indication of the degree of shrinkage was provided.** Consequently, comparisons with measures in the published literature may not be valid. There is no reason to suspect that treatment groups would be differentially affected by tissue shrinkage, but the crucial question of biological significance depends strongly on the actual extent of changes in brain morphometry. Failure to correct for tissue shrinkage will make toxin effects appear smaller in metric units than they really were, if their effects were indeed real.

The study relied entirely on linear measures of thickness of structures as apparent in a few coronal (cross sectional) brain slices. This constrains conclusions, because **thickness measures are sensitive to functionally irrelevant and minor changes in the shape of a structure as well as more important alterations in its overall size** [6, 51]. Measures of area of a structure in a slice are superior, and volume compiled from successive slices is even better for some brain regions. Measuring volume is considerably more labor intensive, whereas measuring area is easily done with several common computer-based morphometry programs and requires only a minor amount of additional time.

Figure 2 is shown on the next page, 5a

For the corpus callosum (CC) in particular, **thickness measures in a coronal section are vastly inferior to a measure of cross-sectional area in a sagittal section**. The latter but not the former is expected to be directly proportional to the number of axons connecting the cerebral hemispheres and their degree of myelination [26, 27, 34]. Thickness, on the other hand, is strongly dependent on precisely where along the anterior-posterior axis the coronal section is made [4, 6, 13]. The front end (genu) and back end (splenium) are relatively thick, whereas the middle portion (truncus) just above the hippocampal commissure is usually quite thin, as shown in Figure 2. Area of the CC can be estimated to an acceptable degree of precision by cumulating thickness measures from a substantial number of slices in succession, but one would need to examine at least 10 such slices [38].

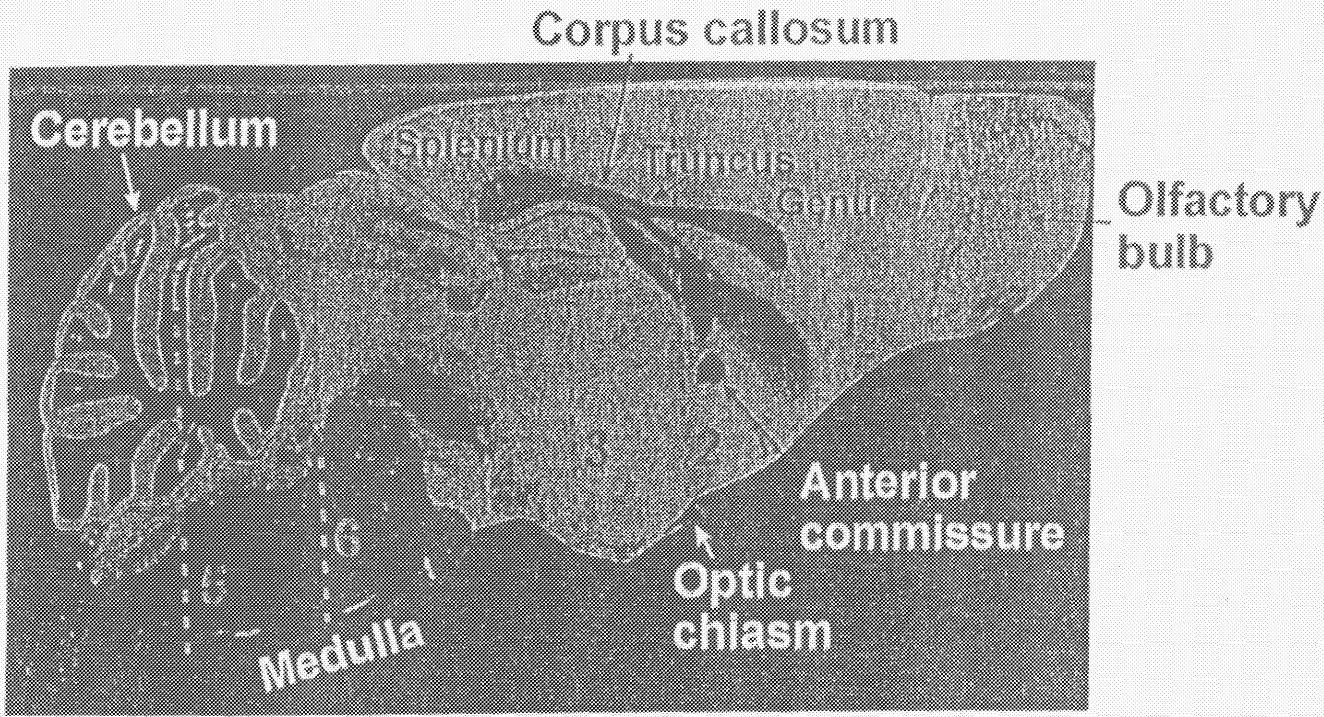


**Figure 2. Sagittal section stained with gold chloride to show myelin.**

Green dotted lines show the approximate location of 6 sections in the study. Note regional variation in thickness of corpus callosum.

For the hippocampus, thickness measures in only one coronal section are of very limited use, whereas areas of CA1 and CA3 provide considerably more information and are commonly used by researchers who study functional aspects of variations in hippocampal size [12, 20, 54].

For the cerebellum, thickness of one coronal section is prone to error because of the angle of the slice and inherent variation in the EGL itself. A sagittal section, on the other hand, lends itself readily to a measure of cross-sectional area of the entire cerebellum and also allows useful measures of the thickness of different zones of the cerebellar cortex [32, 41, 49]. This is especially important when assessing the thickness of the external granular layer (EGL). As the immature granule cells migrate inward past the Purkinje cells, the EGL becomes rather patchy and can have large variations in thickness in adjacent sections. One can adjust to this reality in a mid-sagittal section by taking an average thickness value over an extended segment of the EGL [41] and doing this for several regions of the EGL in different folia.

Thickness of the rat cerebral cortex, on the other hand, is generally an acceptable index, provided thickness is measured at several locations throughout the cortex [28, 40].

Eighteen measures of thickness of brain structures were reported, but 16 of these were measures of only eight structures, where each structure was measured on both the left and right halves of the brain. **Measuring both halves of a bilaterally symmetrical structure is an acceptable practice, but these measures should be averaged for analysis, not analysed separately.** Conducting separate significance tests on symmetrical measures is a practice to be frowned upon, unless the study is specifically aimed at assessing asymmetry and there are good reasons to think asymmetry might be functionally important. In the present study, there is no good reason to refrain from taking an average. Considering measures separately on the left and right inflates the error variance and makes the ANOVA less sensitive to genuine treatment effects, if they are present.

**C. Appropriateness of sampling procedures when looking for thyroid-related effects**

Postnatal deficiency of thyroid hormone (hypothyroidism, sometimes produced by removal of the thyroid gland) is known to increase the size of the cerebellum and the number and complexity of its folia, fissures, and sulci in the rat brain, whereas hyperthyroidism produced by administration of excessive amounts of the thyroid hormone thyroxine generally has the opposite effect [31, 32]. It appears that these effects result from the acceleration and premature maturation of the cerebellum in the hyperthyroid animals, whereas hypothyroidism prolongs the period of maturation, a period during which the numbers of folia and sulci normally increase. These phenomena are readily perceived in a sagittal section through the middle of the cerebellum but are almost impossible to measure in a coronal section.

The number of myelinated axons is substantially reduced in the anterior commissure and corpus callosum of hypothyroid rats [3, 21, 22], and the effect is most apparent in the area of a sagittal section. A reduction of myelinated axons will also reduce the cross-sectional area of the corpus callosum [26, 27], whereas large changes in the numbers of unmyelinated axons may have little impact on corpus callosum area [34]. Recent evidence indicates that thyroid hormones are involved in the maturation of oligodendroglia, cells that are responsible for the formation of myelin in the central nervous system [15].

Hypothyroidism has little effect on the overall size of the hippocampus, but it increases the relative size of one particular component of the hippocampal CA3 region - the mossy fiber projection from the granule cells [31, 33], an effect that is most readily seen in measures of relative areas and especially volumes of anatomical regions of the hippocampus.

It is to be expected that deficiency of thyroid hormone would alter the size and complexity of the dendritic arbor of neurons in cortex and cerebellum [31, 33]. This could be assessed by counting the number of cells in the CA1 and CA3 zones of the hippocampus, for example, and comparing area of the zone with neuron number. This kind of measure is extraordinarily time consuming and requires specialized histological procedures of the highest quality [20, 54]. It is not practical in a large study with many animals in each of many dose and age groups. It would also be important to know whether thyroid hormone effects on corpus callosum size result from changes in axon number or myelination of axons [3, 21, 26, 27, 34], but again the laborious electron microscopy required to make these kinds of observations limits the scope of such a study; even a modest assessment would be very expensive.

**D. Control of sources of inherent variability**

Three sources of inherent variability in measures of thickness were evident in this study. Not one of these three sources of variability was adequately controlled.

First, it was obvious from the thumbnail sketches of coronal sections that **in many brains, especially in the DL10 rats, the section was not cut perpendicularly to the midline of the brain**. This can be seen readily in the section through the most posterior portion of the hippocampus (cut #4 at the level of the mammillary body), wherein there was often a remarkable asymmetry. Consider the very first brain in the report (16616M-2); on the right CA3 is seen in dorsal hippocampus, whereas on the left it extends downward to ventral hippocampus. Figure 2 on page 6 of the Supplemental Morphometry Report shows thickness measures taken in a perfectly symmetrical section, but there must have been many sections where the measures on the left and right were located in substantially different parts of the hippocampus. Averaging values for left and right could have enhanced the sensitivity of the analysis of effects on the corpus callosum by compensating for asymmetry of the section, because those measures were taken close to midline. Alternatively, if the measure on one half appeared to be valid but the other half was rendered dubious because the pertinent structure was too ventral, it would have been reasonable to analyse only the value for the one good half rather than the average of good and bad. I identified 35 brains in the DL10 group where asymmetry was visually obvious in coronal section 2. In almost every instance, the asymmetry was in the same direction, with the portion of the hippocampus on the viewer's left being more ventral. Presuming that the sections themselves were always kept in the same orientation during slicing and mounting on the microscope slide, this suggests a consistent bias on the part of the technician doing the sectioning (something I noticed long ago in my histologist who was rigidly right-handed). I noticed only three instances of asymmetry in the DL22 brains. They had been processed at a different site, and there may have been different levels of care and technical skill in the two labs.

Second, **in many instances, especially in the DL10 brains, the actual anterior-posterior levels of ostensibly the same sections were quite different**, as evident in the thumbnail sketches, and this would have given rise to substantial differences between animals within a group in thickness of the corpus callosum, for example. Only 6 slices throughout the anterior-posterior extent of the rat brain were examined, ranging from a point considerably anterior to the optic chiasm (passing through the anterior septal region) back to the middle of the cerebellum, a distance of over 1 cm. No diagram is provided to the reader to aid judgment of the separation of the measured slices, but descriptions of the 6 sections lead me to suspect they were placed roughly as shown in Figure 2. These estimates are based on my previous studies of mouse brain development [7, 43, 44, 51], making adjustments for the different sizes of rat and mouse brains [7]. Given the limitations of manual methods of sectioning, by far the best means for insuring sections in different brains are taken at the same anterior-posterior level is to slice serial paraffin sections in a long "ribbon" and stain a series of sections in the vicinity of distinctive landmarks. Then the observer can examine the stained slides and select the section in each brain that is closest to the desired level. This is quite impossible when only 6 sections are made.

Third, it is to be expected that a treatment which alters thyroid hormone levels would have an impact on the size of the whole brain, and indeed this appears to have occurred to a modest extent in this study. More important than the modest between-group brain size effect may be the within-group brain size relation with many linear measures. It would be highly desirable to know which effects are specific to the structure in question, rather than being consequences of overall size of the brain. This can be addressed by first adjusting each measure for brain weight of the individual rat and then conducting the analysis of variance [6, 7, 50]. It is expected that **adjustment for brain size would reduce variability within a group.**

Data on DL10 male and female brains (pp. 56, 58) and DL22 male and female brains (pp. 66, 67) suggest that there was somewhat greater variation within a group in the DL10 than DL22 rats for certain measures, and instances of substantially greater variation in certain dose groups were also apparent. The authors ran the Bartlett's test for heterogeneity of variance and indicated no serious problems in this regard. Scatterplots of individual scores would assist the reader to evaluate the likely importance of exceptional scores.

Some of the stained slides were scanned at 1350 dpi rather than 2700 dpi. The smallest structure of interest in the study was the corpus callosum, as represented by thickness measures that were in the vicinity of 300 μm or 0.3 mm, which is about 0.012 inch, the equivalent of 16 dots in the scanned image. This degree of imprecision (1/16) is acceptable, given the presence of other, much greater sources of within-group variation.

The corpus callosum is comprised of axons and a few glial cell bodies, and it is best viewed with a stain that reveals axons or, in the older rats, myelin. H&E gives a general idea of the limits of the CC proper, but in my experience the eosin staining of axon bundles tends to be inconsistent, and the observer tends to rely on hematoxylin staining of neuron cell bodies to decide what regions do *not* contain axon bundles. This source of variability was probably not large in comparison with other sources of within-group variation.

**E. Homology of brain sections**

In many instances there was clearly a **lack of adequate homology**, especially in the DL10 brains, as discussed in section D.

**F. Use of coronal sections in the published literature**

As discussed in section B, **sagittal sections are preferable when conducting morphometric studies of the corpus callosum.** There are some published data on thickness of the CC at midline [13, 38], but I am not aware of studies in which measures were taken bilaterally at the same distance from midline. As one goes further from midline, the size of the corpus callosum is increased by axons that travel to and from cingulate cortex but remain in the ipsilateral cortex without crossing midline [24], and thickness involves more than the corpus callosum proper.

Dorsal hippocampus is best studied with the aid of coronal sections, whereas **ventral hippocampus requires the use of horizontal sections** to visualize the sizes of CA1, CA3, and anatomical layers. Probably the best morphometric data in the literature involve horizontal sections [12, 33].

Direct comparisons of thicknesses in terms of microns (μm) is rendered difficult by the use of paraffin embedding that causes substantial tissue shrinkage. This is a problem mainly for the DL22 rats at an age when frozen sections are perfectly adequate. The more delicate tissue of DL10 brains makes paraffin embedding a reasonable choice, although we have obtained very good results by encasing the brains in gelatin and then slicing frozen sections [43].

**G. Comparisons of thicknesses with the published literature**

Given the idiosyncratic methods employed in this study and the specific ages examined, comparisons with the published literature are difficult. The rat brain is changing rapidly during the DL10 to DL22 period, and it would be important to compare brains no more than one or two days different in age. The authors should have addressed this matter during the proposal and design stages and then again in the discussion of the results to cite standards for comparison. **I was surprised to find no references to any published literature on the rat brain in the entire report.** There may well be some comparable published values, and if time allows I will make a search for them. At this point, all I can say is that the stained tissue in the thumbnail sketches looks like the kind of histology I would expect to see. Nothing strikes me as being remarkably abnormal, but this opinion warrants little weight and might be different if sagittal sections were available.

**H. Limitations of data confined to DL10 and DL22**

As discussed in section A, **data on more mature rats would aid interpretation**, because rapid changes in brains at DL10 and DL22 make the results sensitive to changes in rate of growth as opposed to later asymptotic levels. Myelination of the CC in particular is rudimentary at DL10 and far from complete at DL22, although major anatomical changes brought about by elimination of axons are past [16, 25]. If one were to find effects of a toxin at DL10 but not

DL22, conclusions would be quite different than if effects were seen at both DL22 and DL100, for example. Use of multiple time points would add to the expense of a study, so the crucial question is which time points are most important to examine. In my opinion, the most critical issue is whether a toxin has lasting effects, so one would certainly want to see data from rats that have reached reproductive maturity.

**Issue 2: Biological significance of results**

**I. Consistency of effects on brain development**

The question of consistency first brings us to the question of statistical significance. There are two kinds of errors that can be made when drawing inferences from group comparisons about treatment effects. There may be no real effect of a substance in the drinking water, yet random assignment of rats to groups may produce group differences in means that are large enough to prompt us to reject the null hypothesis of no effect. This would amount to a false positive result or a Type I error. It is customary to set a criterion for Type I error at some fixed value $\alpha$ at the outset of a study. The proper level for $\alpha$ is a matter of considerable controversy in the literature. Use of $\alpha = .05$ (one false positive per 20 independent tests) is commonplace, but in studies where many tests of significance are done, one sometimes finds $\alpha = .01$ employed, and in my field where genetic linkage analysis is done, $\alpha = .0001$ is often recommended [29]. Both .05 and .01 values are cited in the present study.

The second kind of error (Type II error) can occur when there is a genuine treatment effect, but the statistical test fails to reveal it because the test lacks sufficient power [45, 46, 47]. This kind of result is most common when the treatment has generally small but real effects, because a small signal is less likely to be heard in the presence of background noise. In toxicology, two general patterns might occur. There might be very clear, even obvious effects at high doses, in which case the main challenge will be to ascertain the no-effect dose. The thyroid hormone data exemplify a clear dose-response relation. On the other hand, there may be some doubt as to whether there is any consistent effect at all, at any dose, especially if small or modest samples are examined. This latter pattern is the point at issue in the Primedica study in so far as morphometric data are concerned.

This study assesses consistency of effects by comparing each dose with a zero-dose control using a Dunnett's test. There are two shortcomings to this method. First, when multiple comparisons with a single control are made, a more stringent criterion for significance of any one comparison is used than for a simple t test comparing two groups, and this reduces the power of the test and increases the probability of a Type II error. Second, the test remains vulnerable to a spuriously low or high mean value of the zero-dose control. This may well have occurred in the DL10 females, where controls exceeded the 0.01 dose on almost every measure, although the differences were usually not significant at $\alpha = .05$. There was also a tendency for DL10 females receiving the 1.0 dose to exceed both the 0.1 and 30.0 doses for many measures. How could there be such a consistent pattern of group differences and yet such a low frequency of significant differences? Within a group, the many measures come from the same animals, and a group that tends to have an unusually high mean brain weight will also tend to have relatively high scores on all measures. That is, **the many tests of significance conducted on different**

**measures in this study were not themselves independent assessments.**

The linear regression analysis presented in a separate section is a step in the right direction, in that it asks whether there was a tendency for effect to increase with dose. This kind of test, being based on the pattern across five groups rather than a comparison of only two, should be less sensitive to anomalously high or low scores in any one group. Scanty details of the analysis made it very difficult to judge, however. It is not clear to me whether the analysis used individual animal scores or group means for the linear correlation with dose. It is also not clear whether the regression used log or untransformed dose. The plots of means were obviously done with a log dose scale, but comments on the first page of the regression analysis suggest log dose was not used for the regression. Note the authors' remark that "..a curve that appears linear in a figure might not represent a true linear dose response." This suggests the "true linear" response was assessed by regression analysis without a log transform. The figures cite "R" values and P values, P being probability of Type I error, but degrees of freedom are not specified. Capital R is usually the symbol for the multiple correlation coefficient, but multiple R is appropriate only when there are two or more predictors. In this analysis where they purport to assess linearity, however, there can be only one predictor, X = dose. Hence, we would expect to see Pearson r. Examining the first graph of Left CA3 thickness of DL10 males, the linear relation with dose is described as R = 0.902, significant at P = .036. Taking the mean scores from the table on page 56, shown in Table 2, I calculate that the Pearson r = 0.902 with 3 degrees of freedom. My tentative conclusion is that the R values given on the dose-response plots in the report are actually Pearson correlation coefficients computed on group means and having a pathetically low 3 degrees of freedom. If so, the analysis was not a regression analysis in the usual sense and was quite inappropriate. In this analysis, the dose at X = 30.0 is an outlier and all the other three doses are huddled close to 0.0 (see Figure 3), so in any case where the group mean for the 30.0 g/Kg/day dose happened to be quite a bit higher or lower than the other four groups combined, Pearson r would tend to be high. The tests done in the "regression" analysis simply reflected the difference between the mean of group V (30.0) and the mean of the means of the other four groups, and they did not properly assess any linear dose trend. **This kind of test would not be capable of revealing a moderate linear effect across the dose range with reasonable statistical power. Instead, it was sensitive only to exceptionally large effects of the highest dose.**

**Table 2 CA3 mean thickness (Y) versus untransformed dose (X) for DL10 male rats**

| | | | | | |
|---|---|---|---|---|---|
| X: | 0.0 | 0.01 | 0.1 | 1.0 | 30.0 |
| Y: | 274 | 285 | 271 | 282 | 304 |

**The analysis of data in this report was not adequate to allow me to evaluate the consistency of perchlorate effects on any measure.** A fair number of significant deviations from the zero-dose control were detected, particularly for male rats, so it would be imprudent to dismiss the report as failing to demonstrate any effect on brain morphometry. Examining the means in the tables on pages 56, 58, 66, and 67 suggests to me that perchlorate effects on morphometric measures were not large, with the possible exception of cortex and maybe hippocampus of DL22 males. Given my reservations about the statistical methods, it would not be wise to substitute "eyeball" impression for a proper analysis.

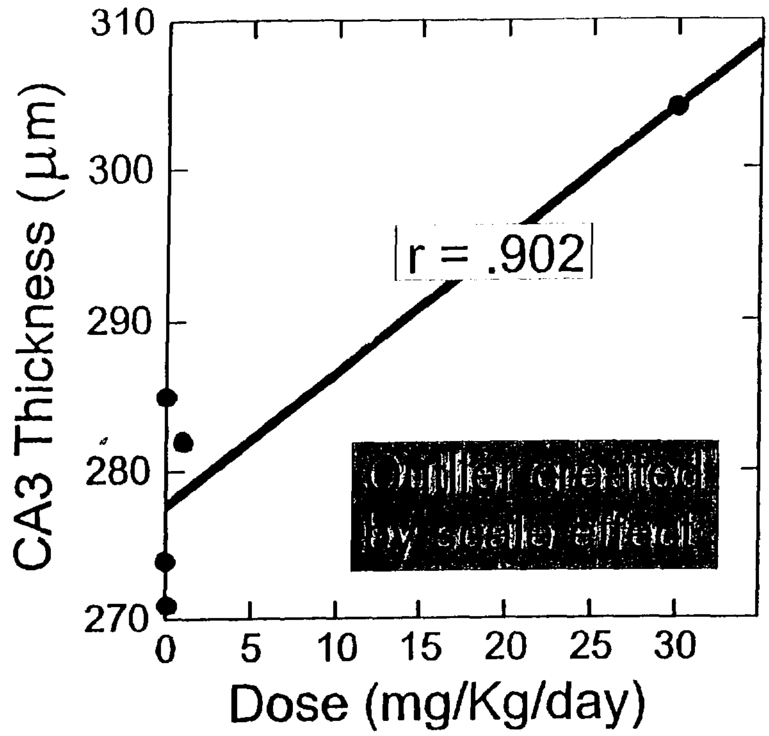


**Figure 3. Plot of data used for "regression analysis"**

**J. Anomalously high scores at 1.0 mg/Kg/day and low scores at 30.0 mg/Kg/day**

An intermediate dose could certainly have the maximum effect when there is some kind of biphasic dose-response relationship on certain measures, but it could also be spurious. The proper test of a U-shaped dose-response curve is the quadratic term in a regression equation, assessing whether addition of an $X^2$ term signficantly improves the fit of the equation to the data. No such test was reported.

**K. Sex difference in direction of perchlorate effects on morphology**

As discussed in section A, a piecemeal approach to data analysis whereby separate tests of effects are done for males and females is not an adequate basis for judging a sex difference in response. The results presented in this manner hint at a sex difference in response but do not present sufficient statistical evidence to persuade. In particular, I see **no clear evidence that there was a decrease in thickness measures with increasing dose for females**. There is reason to suspect that the so-called decrease in DL10 females was an artifact of an anomalously high mean of the zero-dose controls, as discussed above. In DL22 females, some dose group was significantly ($\alpha = .05$) higher than controls for the striatum, CA1 and CA3, but significantly lower than controls for cerebellum and corpus callosum #2. It is possible that a proper statistical analysis would reveal a significantly greater perchlorate effect for males and females, but the present report provides no such evidence. Lacking compelling statistical evidence, I can see no point in delving deeply into the physiology of the sexes.

**L. Biological significance of changes in linear dimensions**

As mentioned earlier, linear dimensions are not good indicators of biological function; area and volume measures are expected to be more closely related to function. Nevertheless, the authors of the study must have believed that thickness measures are valid indicators of potentially harmful perchlorate effects. The question to be answered is therefore: How great a change in thickness should be required to support a claim of harm or benefit to the organism? This is a matter that should be agreed upon in advance by the sponsors and consumers of the research, and it should play the crucial role in deciding the sample size in order to confer sufficient power on the test.

I strongly recommend that the term "significance" not be used in this context, given its clear, technical meaning as the probability of a Type I error in the scientific report. Conflation of statistical and biological, psychological, or social significance can lead to serious misinterpretation of research by scientists and the public alike [11, 45, 47]. For this reason, statisticians have devised separate indicators of the magnitude or size of a treatment effect, and these indicators provide guidelines for deciding on the presence of a biologically important, substantial, or noteworthy effect.

Most of the widely accepted indicators of effect size compare the difference between treatment and control groups with the variation within a group. For example, effect size d is the ratio of the difference between group means to the standard deviation within the groups; $d = (M_1 - M_2)/S$. In psychological research with humans, small, moderate and large effects are equivalent to d = 0.25, 0.5 and 0.8, respectively [10, 11]. I have argued that in laboratory work with animals where we can control many factors so much better, small, moderate and large effects are shown by d = 0.5, 075, and 1.0, respectively [47].

Another index of effect size is the coefficient $\omega^2$, which tells us the proportion or percentage of the total variance in scores of all subjects in the two groups that is attributable to the difference *between* the group means; $(1 - \omega^2)$ is then the proportion that resides *within* the groups and is unrelated to the treatment. When sample sizes are reasonably large, there is a simple relation between the two indicators of effect size: $\omega^2 = d^2/(d^2 + 4)$. This relation is expressed in Table 3.

**Table 3 Standards for small, moderate, and large treatment effects**

| Effect size: | Small | Moderate | Large | Very large |
|---|---|---|---|---|
| d | 0.5 | 0.75 | 1.0 | 2.0 |
| $\omega^2$ | 0.06 | 0.12 | 0.20 | 0.50 |

If instead we are interested in the correlation between two continuous measures, such as size of the brain and thickness of a brain region, or dose of a toxin and test score of an animal, Pearson correlation (r) is often calculated, and the corresponding effect size, comparable to $\omega^2$, is $r^2$, a ratio that tells us the proportion of variation in Y that can be accounted for by knowing the individual's value on X. In order to account for half of the variation in some dependent variable, the predictor should yield a Pearson r = 0.707 to be judged very large by the same standard used to judge $\omega^2$.

Statistical significance, as summarized in the P or $\alpha$ value, is only the first, small step in the process of making an inference, a step that merely decides whether we have made it through the gateway into serious discussion about toxicity. If a difference is not statistically significant ($P > \alpha$), then we have no grounds to estimate $\omega^2$ or $r^2$. Once we have decided that an effect is indeed "significant" on the basis of the statistical test, then we are entitled to inquire about how large the effect appears to be.

**The report cites numerous P values but gives no indicators of effect size.** From tables showing means and standard deviations, it is possible to compute d with a calculator, but this is a messy procedure because the estimate of standard deviation should be based on all five groups (I to V), not just on the two being compared, and this is a slow process with a hand calculator. Let us turn to the table on page 66 for the DL22 males and focus on data for frontal and parietal cortex. For frontal cortex, the standard deviation is (very) roughly 70 µm and the difference between means of the control and highest dose group is also roughly 70 µm, so the effect size is roughly $d = 1.0$, and this would be considered a large effect. A similar effect size is evident for partietal cortex.

I want to emphasize that standards of effect size based on d, $\omega^2$ or $r^2$ are those deemed appropriate for basic neuroscience and may not necessarily be the best metrics for toxicology research. Consider the meaning of $d = 1.0$. In the case of parietal cortex, this tells us that a perchlorate group had a cortex about 70 µm thicker than the controls. At the same time, we should expect that animals *within the untreated control group* would have a range of cortical thicknesses of about four standard deviations or 280 µm, and some of them would be likely to have thicknesses greater than the mean of a perchlorate group; likewise, some of the perchlorate-treated rats would be below the mean of the untreated controls. This is confirmed from raw data shown in tables in the detailed neuropathology reports. Only when group means differ by almost four standard deviatons ($d = 4.0$) would we expect to find no overlap between scores of the controls and treated animals [47, 50]. One fruitful approach to setting a standard for noteworthy effect size in toxicology would be to review data from research on other substances at doses that are generally agreed to have reliable effects. Effect size indicators have no units of measurement, and the review could encompass research that did not even study the brain. A review of this nature is beyond the scope of the present report.

Biological importance should also be judged relative to possible effects on the size of the whole brain, because the judgment about toxicity might be somewhat different if there were an overall effect on organism or organ growth versus an effect specific to a certain structure. Looking at the same table for DL22 males, it appears that the perchlorate effect on brain size was close to $d = 1.0$ for the 1.0 mg/Kg/day dose and somewhat smaller for the other doses. Quite a few other variables show an increasing trend, as detected by my eyeball, so there might very well be an overall brain size effect. Lacking a proper regression or linear trend analysis, however, we should be cautious about drawing conclusions and vigilant against the possibility of an anomalously low brain size of the controls. A proper regression analysis is possible with the data in hand, but interpretation will be clouded by the fact that brain weights were based on formalin-fixed tissue prior to paraffin processing, whereas thickness measures were based on seriously shrunken brains. This discrepancy would not necessarily invalidate an analysis, but it would make interpretation more difficult. The prospects for a re-analysis of the data are discussed further in section T, the Overall Evaluation of the study.

Another approach to judging biological importance would be to compare the present results for cortical and corpus callosum thicknesses with similar measures from a separate study of toxic effects on rat brain growth involving a substance that is generally agreed by all parties to be harmful. One would need to be sure that tissue was processed and measured similarly in the two studies, of course. I am not aware of published studies that are directly comparable in this way.

**Issue 3: Biological plausibility of results**

**M. Linear dimensions versus thyroid effects**

I am not qualified to evaluate changes in thyroid histopathology.

**N. Correspondence with severe hypothyroidism**

My knowledge of the literature on severe hypothyroidism in humans is not sufficient to provide an expert opinion.

**O. Mediation of effects by thyroid hormone levels**

On this topic, there is a considerable literature published on the artificial administration of thyroid hormones to developing rats and reduction of thyroid hormones by removal of the thyroid gland. Although I am familiar with a limited range of this literature, it is my understanding that thyroid hormone administration commonly changes brain size and the linear dimensions of many brain structures. Most of these studies, however, involve hormone administration during a relatively narrow, usually postnatal age range, whereas the Primedica study involved perchlorate treatment starting well before conception and continuing throughout life. We now know that thyroid hormones have important effects on prenatal brain development [14, 37]. A great deal is known in general about thyroid biochemistry and physiology [2, 9, 37], including mechanisms by which thyroid hormones affect axon growth and myelin formation [1, 15]. Thyroid physiology from a developmental perspective is rather complex, and one cannot state with confidence that hypothyroidism will always decrease some anatomical feature or that hyperthyroidism will increase size. As pointed out in the Primedica report, there may also be physiological compensation in a long-term study. In this study, the TSH levels were consistently increased by perchlorate in a dose-related manner, but circulating thyroid hormone levels were decreased. I am not qualified to give an expert opinion on the developmental impact on the brain of this kind of biphasic response.

**Issue 4: Relationship with behavioral/functional effects**

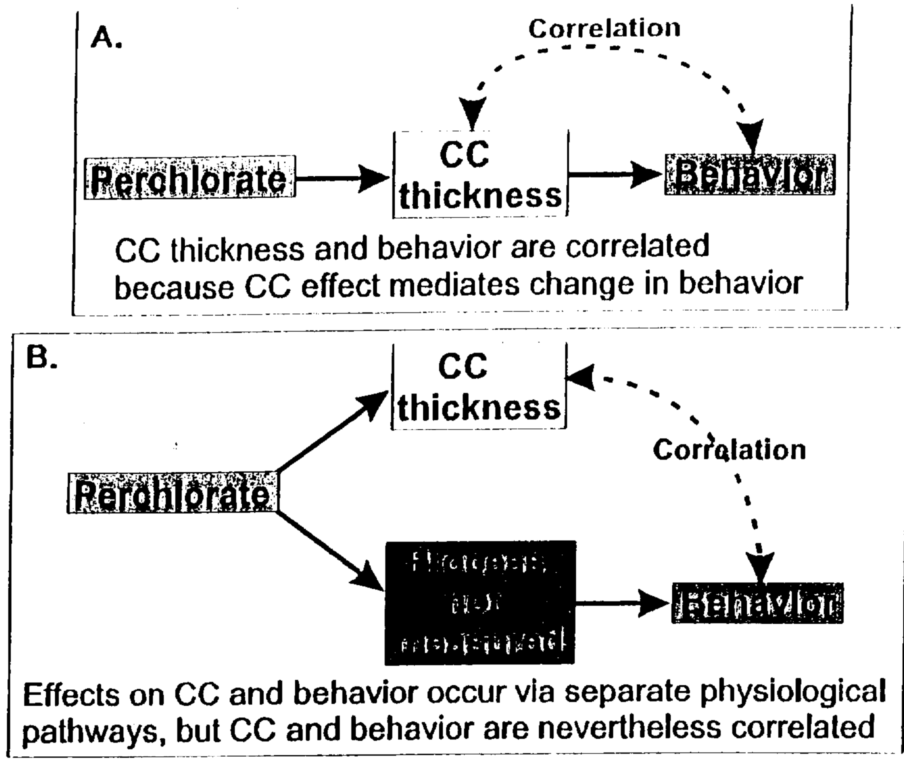


**Figure 4**. Two scenarios that could produce a CC:behavior correlation. A. CC mediates perchlorate effect of behavior. B. Effect on CC and behavior occur via different pathways

**P. Association of thickness changes with behavioral effects in adults**

This is a very interesting but difficult question. The principal challenge is to establish a causal connection between the two things. Of course, in a carefully controlled study, and the Primedica study in its execution did appear to be well controlled, consistently significant effects of perchlorate must have some causal relation to outcome. At the same time, the present study did not assess behavior or any other biological function, except body growth of the pups. So, for purposes of discussion, let us suppose that a study observed significant and large effects on *both* cortical or CC thickness and several behaviors. Would this provide proof that thickness mediated the changes in behavior? As suggested in Figure 4, this conclusion would not necessarily follow from the evidence. In Figure 4A, the hypothetical perchlorate effect on behavior is mediated by the change in thickness, whereas in Figure 4B both thickness and behavior are altered by perchlorate treatment, but the effects are exerted via separate, unrelated physiological pathways. In both cases, there would be a consistent correlation between change in thickness and behavior. I make this point in order to clarify the question asked about association. When association is the principal concern, I surmise that those interested in the answer are not particularly concerned about the issue of mediation or causal pathway. That is, thickness measures might be taken as important indicators of a potentially important functional change, even though thickness itself might be seen an epiphenomenon of no special functional relevance.

What this question boils down to, in the context of rat brain morphometry and behavior, is whether changes in whole brain size or corpus callosum size are likely to have noteworthy effects on behavior. This has been a vexatious issue in the literature of behavioral neuroscience for several decades, and no general conclusion can be cited. It has been very difficult to demonstrate any consistent behavioral consequence of a change in whole brain size, even though many treatments are know to alter brain size. Even selective breeding for high or low brain size cannot be relied on to change behavior in a predictable direction [23]. In the literature of human neuropsychology, many claims have been staked on a relation between brain size and intelligence or IQ, but the data point to a very small correlation with almost no explanatory impact [44].

Likewise for corpus callosum size - it has been very difficult to demonstrate any reliable behavioral correlate of modest changes in CC cross-sectional area. In my laboratory and others, it has been found that mice lacking any CC whatsoever are reasonably normal on a wide range of tests [5, 8, 39], with the possible exception of those involving complex motor movements performed at high speed [39]. Surgical section of the CC in adults produces a cerebral disconnection (split brain) effect that is well known, but the same deficit does not occur when there is failure of CC formation in the human embryo. Humans lacking a CC from the beginning of cortex formation tend to be a bit clumsy and are on average about 10 points below average on IQ tests, but they almost always suffer other neurological challenges, including neuromuscular degeneration, and there is no indication that reduced cognitive functioning arises specifically from the lack of a CC [30].

The literature on variation in CC size within the normal range in humans is controversial. Early in the 20$^{th}$ century it was claimed that men with a large CC are exceptionally intelligent, but that hypothesis has not been confirmed. In 1982 it was claimed that the posterior portion (splenium) of the CC was relatively larger in women than men and might form a basis for cognitive gender differences, but a comprehensive review of 49 published studies on the topic found that men have larger brains than women (on average, $d = 1.1$) and slightly larger CC area ($d = .2$) without any consistent sex difference in CC shape [6]. There is some evidence of a relation between the size of a central portion (isthmus) of the CC and handedness in humans [6].

Numerous studies in rats have found a sex difference in overall CC size and a variety of steroid hormone effects on the CC [13, 17, 18, 19, 35, 36]. We have shown that the CC size effects are consistent with the sex difference in whole brain size [7, 50], but we do not claim that experimental hormone treatments are necessarily mediated by changes in brain size.

My conclusion from this large and difficult research literature is that **one would not expect any major change in behavior to arise from a modest change in the thickness of the CC in one or two coronal sections.** I would not recommend dismissal of a thickness change as irrelevant, however. It is possible that **a change in thickness might serve as an indicator of a more functionally important change elsewhere in the nervous system.**

**Q. Appropriateness of behavioral endpoints**

I was asked to evaluate the Primedica study, and there were no behavioral endpoints therein.

### Issue 5: Relevance to humans

### R. Generalization from rats to humans

Animal models are crucial throughout the field of neuroscience and neurotoxicology, and the question of generalization across species is exceedingly difficult. I am aware that this matter has been debated for decades by experts in toxicology and public officials responsible for regulating use of thousands of compounds in industry and medicine. The only wisdom I might possibly add here involves comparisons of mice and rats. These two rodents are very similar, to the extent that a stained mouse brain slide magnified a little looks remarkably like rat tissue, and they are close relatives on an evolutionary time scale. Nevertheless, the corpus callosum of many rat strains shows a definite sex difference, whereas for many mouse strains the CC of males and females is almost identical in size, as are their brains, too [7]. So, which species is the better model for humans? I will abstain from this vote. If researchers find that a compound has consistent effects across a wide range of mammalian species, then I think we can be confident that results will generalize to humans, but I would not care to generalize on the basis of data from any one species. If toxic effects are observed in rats, this would certainly provide grounds for worrying about possible harm to humans.

### S. Appropriateness of rat brain morphometry as a model for human neurodevelopment

I am not at all concerned about the transfer of principles of morphometry across mammalian species. Corpus callosum, cerebellum and hippocampus can be measured the same way in both rats and humans, as can almost every other brain structure. The elaborate gyrification of the human cerebral cortex makes measurements of cortical thickness more challenging than in the rat with its smooth cortex, but excellent stereological methods to address this problem are available. Species differences in development may be important when we want to match phases of brain growth. This is fairly easy to accomplish in embryos and fetuses but becomes more difficult as the individuals mature. In mouse genetics, we are still debating when a mouse becomes an "adult" and when it is an adolescent. Humans are born with their eyes and ears working quite well, whereas rats have their eyes and ears sealed shut until about two weeks after birth. Humans require many years to progress from weaning to sexual maturity, whereas rats can breed only a few weeks after weaning. The DL10 rats in the Primedica study had just begun to benefit from myelination of the corpus callosum, a stage that occurs prior to birth in humans. The DL22 rats were only a few days from weaning, something that commonly occurs one year after birth in humans but can be delayed for several years for social reasons. There were no rats tested at or after the age of sexual maturity in this study; hence, the hot topic of when teenage boys and girls become adults is moot.

### T. Overall evaluation

In my opinion, the design and execution of the Primedica study up to the point where brain histology was done were adequate and in some ways superior, especially with regard to the long term administration of the perchlorate. Morphometry based on thickness measures in coronal sections is acceptable for judging thickness of the cerebral cortex and informative but less than ideal for assessing the hippocampus, whereas thickness in one or two sections is not acceptable for evaluating changes in the size of the corpus callosum or cerebellum. By far the weakest part of the study was the statistical analysis of the data, which in most respects was well below the standards of what is acceptable in the field of behavioral neuroscience. A proper statistical analysis of the existing data could be done to assess perchlorate effects. Lacking such an analysis, **I cannot form an opinion on the central issue of whether perchlorate significantly altered brain morphology or what may be the minimal effective dose.**

It is also my opinion that **a better statistical analysis should be done for certain measures that appear to have been made with reasonably good methods.** A great deal of effort and considerable expense were devoted to the Primedica study, and I think it would be worthwhile to extract as much meaningful information from it as possible. Specifically, measures of cortical thickness were adequate and measures of hippocampus were somewhat informative, and these could be re-assessed statistically using the raw data provided in Tables 1 and 2 for the DL10 and DL22 rats. Wherever possible, measures on the left and right should be averaged. It would also be important to check each scanned brain section for asymmetry of the cut and tissue quality (see section D), using only the best value on one side if the other was of doubtful quality. The measures of corpus callosum and cerebellum, on the other hand, were so inferior to what ought to be done in a morphometric study that a re-analysis is probably not worth the time.

Once averages of data on the left and right have been used in a multiple regression analysis and data have also been adjusted for linear relations with whole brain size, **a much clearer picture of perchlorate effects in the dose range used in the Primedica study would emerge from a re-analysis.** What that picture would show is something I cannot anticipate in advance.

Signed,

MusWare Technology Inc.

Per ______________________
Douglas Wahlsten, Director

Date April 12, 2001

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